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(54) **IMMUNORECEPTOR MODULATION FOR TREATING CANCER AND VIRAL INFECTIONS**

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(57) **ABSTRACT**

A method of reducing or relieving immune inhibition in a mammal includes the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal. Typically, inhibiting or reducing CD96 activity does not include, or depend upon, killing of CD96-expressing cells in the mammal. The method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent cancer or cancer metastasis and/or a viral infection in the mammal. Also provided is a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent that relieves immune inhibition and/or enhances or restores immune surveillance in a mammal. Typically, the CD96-inhibitory agent is an antibody or antibody fragment.

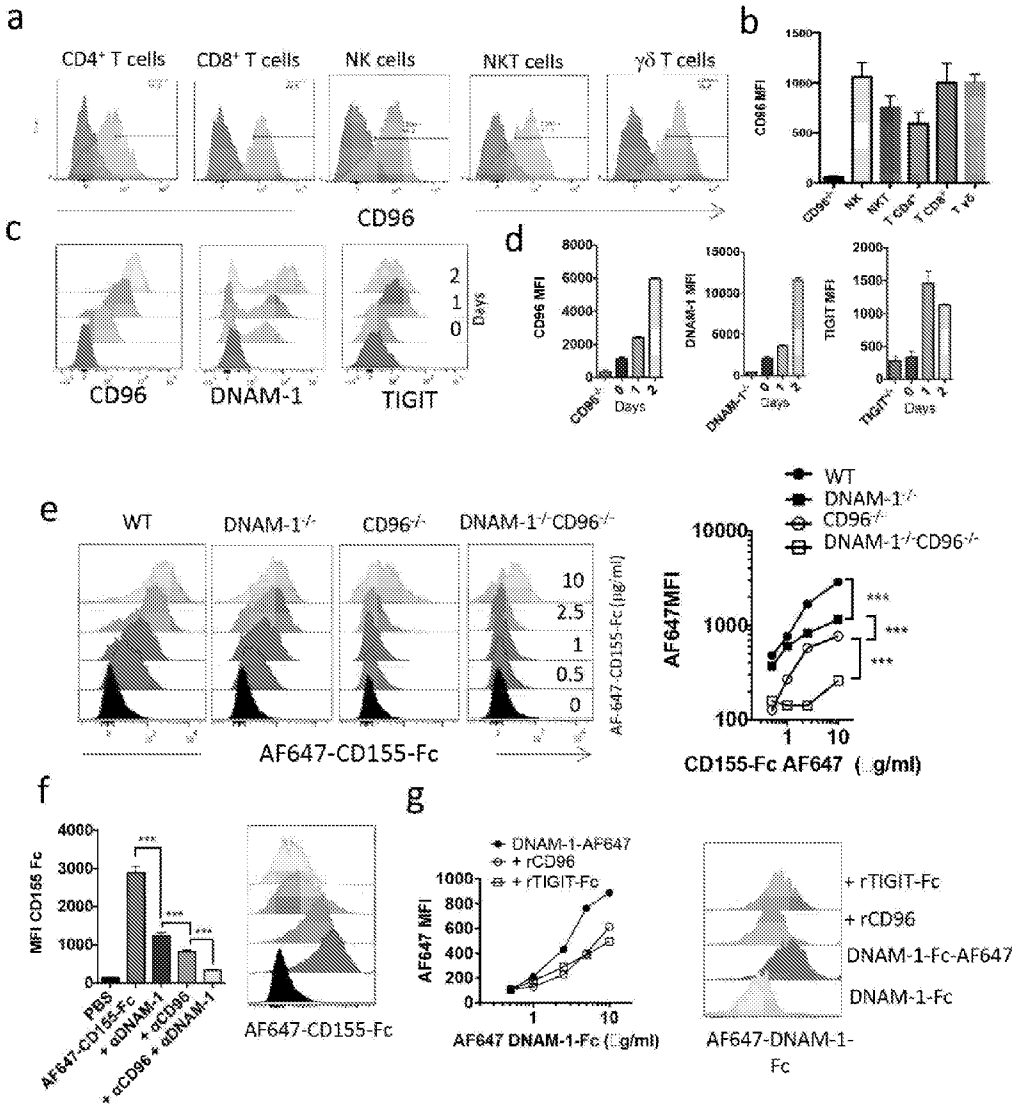

a
CD4+ T cells
CD8+ T cells
NK cells
NKT cells
γδ T cells
CD96
b
CD86 MFI
c
CD96
DNAM-1
TIGIT
Days
d
CD96 MFI
DNAM-1 MFI
TIGIT MFI
Days
e
WT
DNAM-1-/-
CD96-/-
DNAM-1-/-CD96-/-
AF647-CD155-Fc
AF-647-CD155-Fc (μg/ml)
AF647MFI
CD155-Fc AF647 (μg/ml)
f
MFI CD155 Fc
PBS
AF647-CD155-Fc
+ αDNAM-1
+ αCD96
+ αCD96 + αDNAM-1
AF647-CD155-Fc
g
DNAM-1-AF647
+ rCD96
+ rTIGIT-Fc
AF647 MFI
AF647 DNAM-1-Fc (μg/ml)
+ rTIGIT-Fc
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
AF647-DNAM-1-Fc

Fig. 1

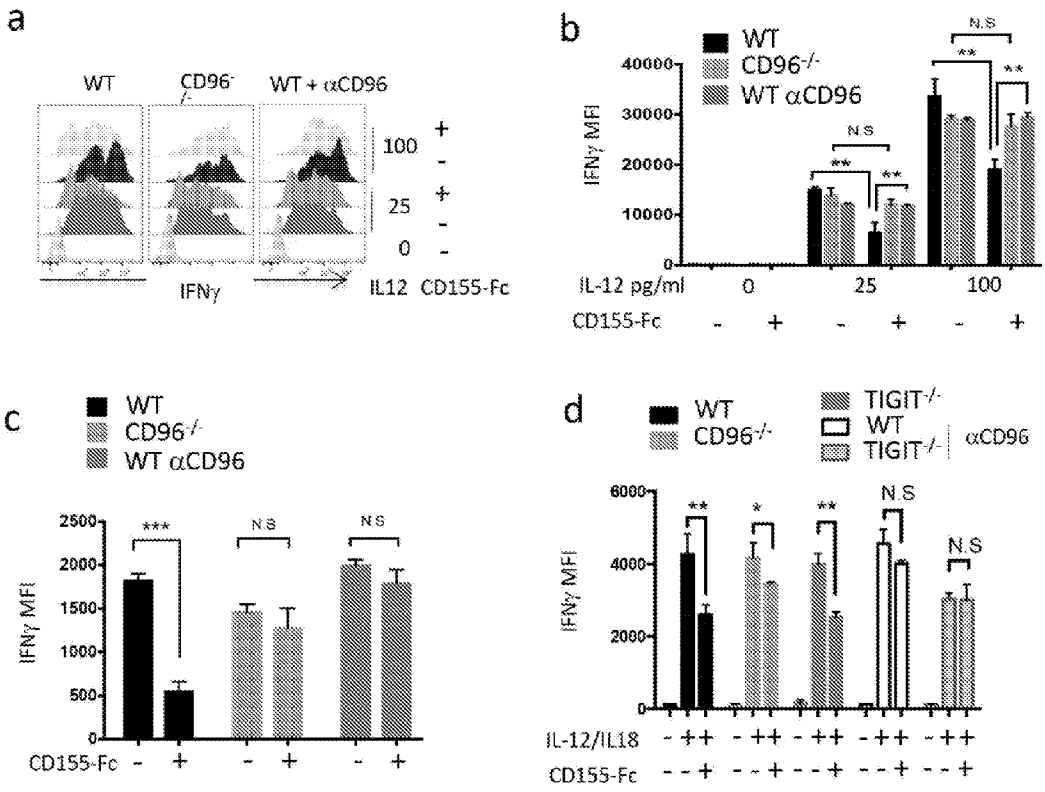

a
WT
CD96-/-
WT + αCD96
100
25
0
+
-
IFNγ
IL12
CD155-Fc
b
WT
CD96-/-
WT αCD96
IFNγ MFI
N.S
**
IL-12 pg/ml
0
25
100
CD155-Fc
c
WT
CD96-/-
WT αCD96
IFNγ MFI
***
N.S
CD155-Fc
d
WT
CD96-/-
TIGIT-/-
WT
TIGIT-/-
αCD96
IFNγ MFI
*
N.S
IL-12/IL18
CD155-Fc

Fig. 2

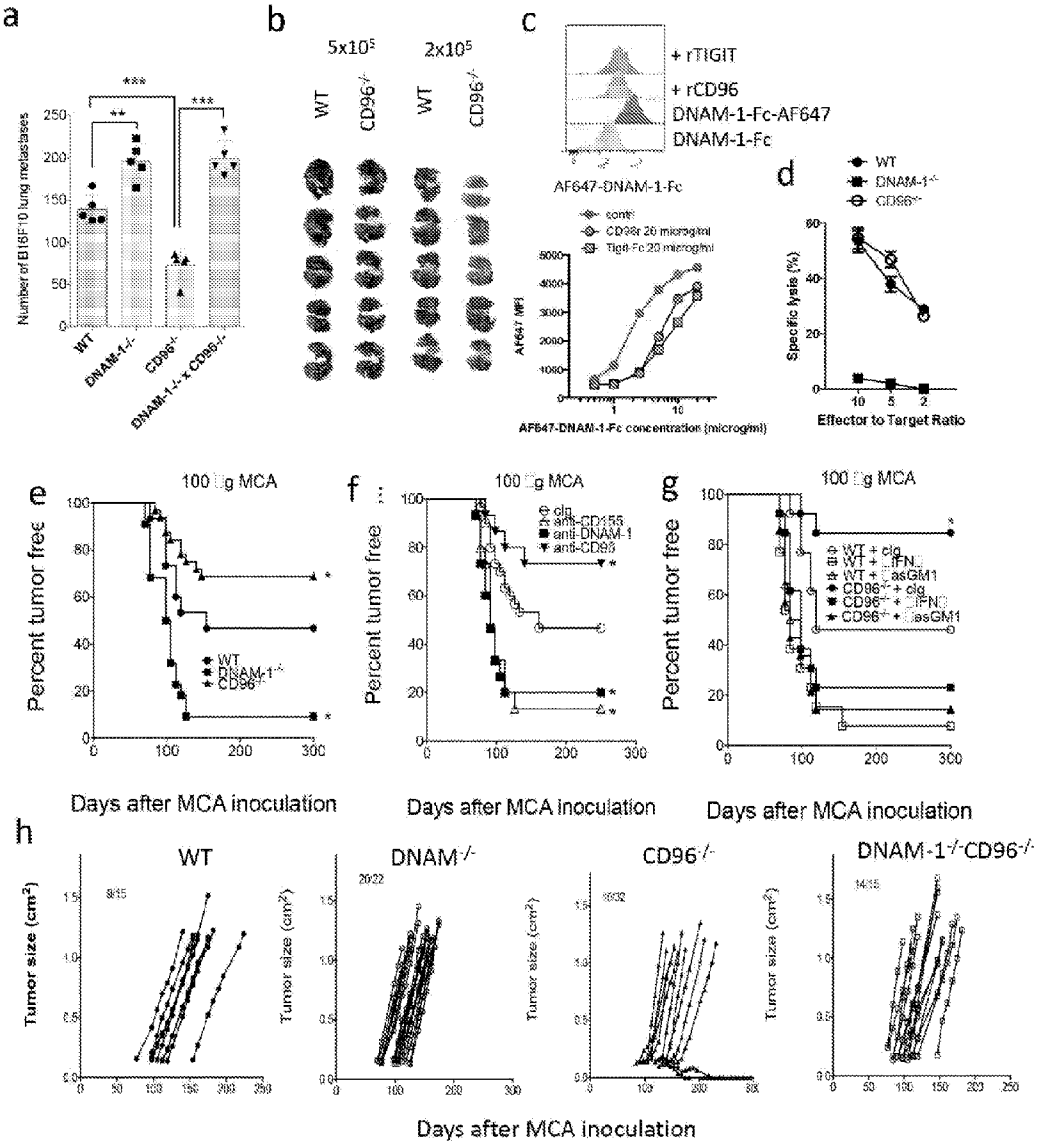

a
b
c
d
e
f
g
h
Number of B16F10 lung metastases
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
5x10^5
2x10^5
+ rTIGIT
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
AF647-DNAM-1-Fc
AF647-DNAM-1-Fc concentration (microg/ml)
Specific lysis (%)
Effector to Target Ratio
100 µg MCA
Percent tumor free
Days after MCA inoculation
anti-CD155
anti-DNAM-1
anti-CD96
Tumor size (cm²)
DNAM-1-/-CD96-/-

Fig. 3

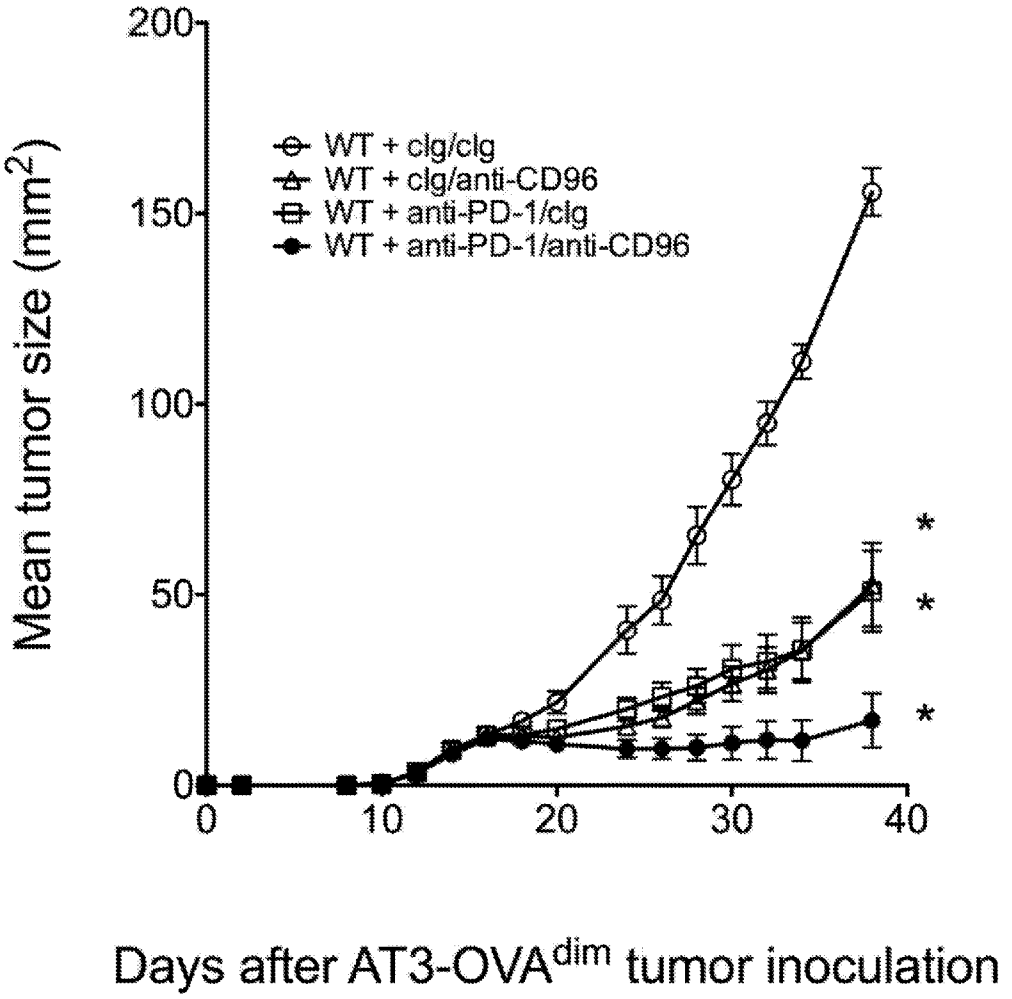

200
150
100
50
0
Mean tumor size (mm2)
WT + clg/clg
WT + clg/anti-CD96
WT + anti-PD-1/clg
WT + anti-PD-1/anti-CD96
*
*
*
0
10
20
30
40
Days after AT3-OVAdim tumor inoculation

Fig. 4

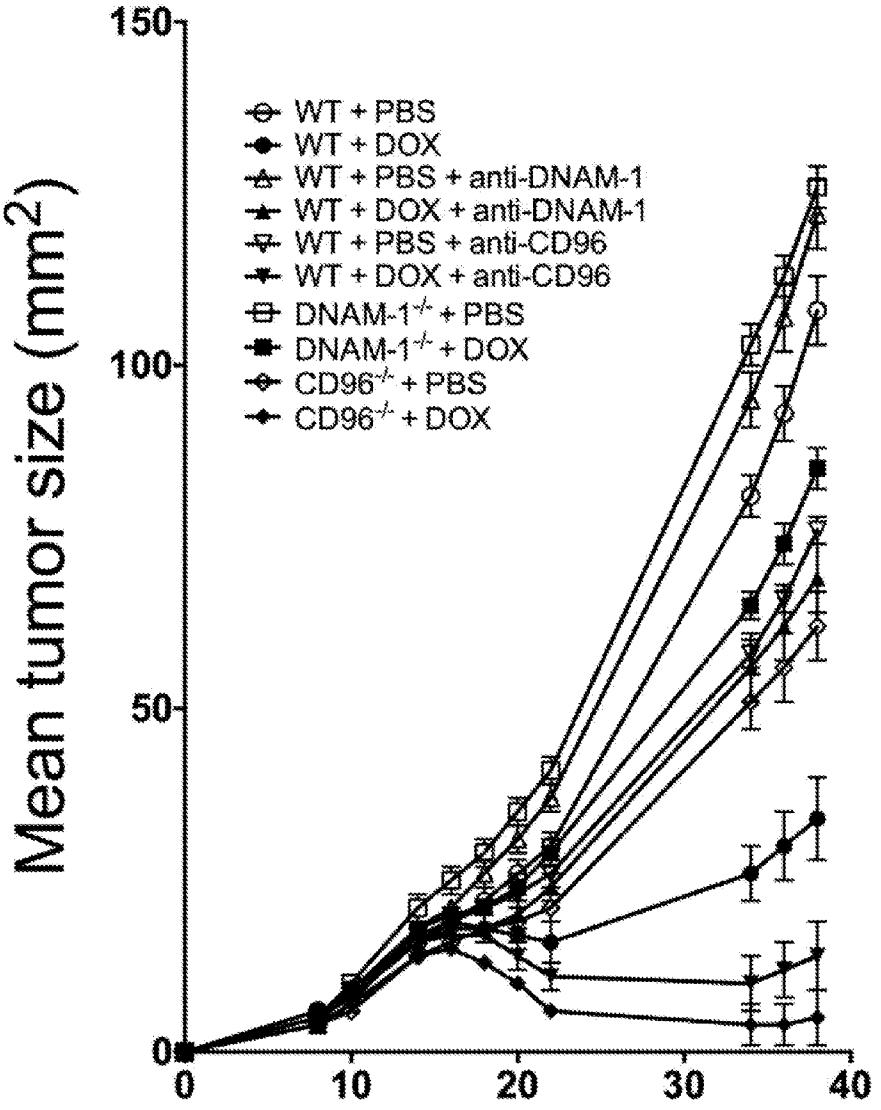

150
100
50
0
0
10
20
30
40
Mean tumor size (mm2)
WT + PBS
WT + DOX
WT + PBS + anti-DNAM-1
WT + DOX + anti-DNAM-1
WT + PBS + anti-CD96
WT + DOX + anti-CD96
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
Days after AT3-OVAdim tumor inoculation

Fig. 5

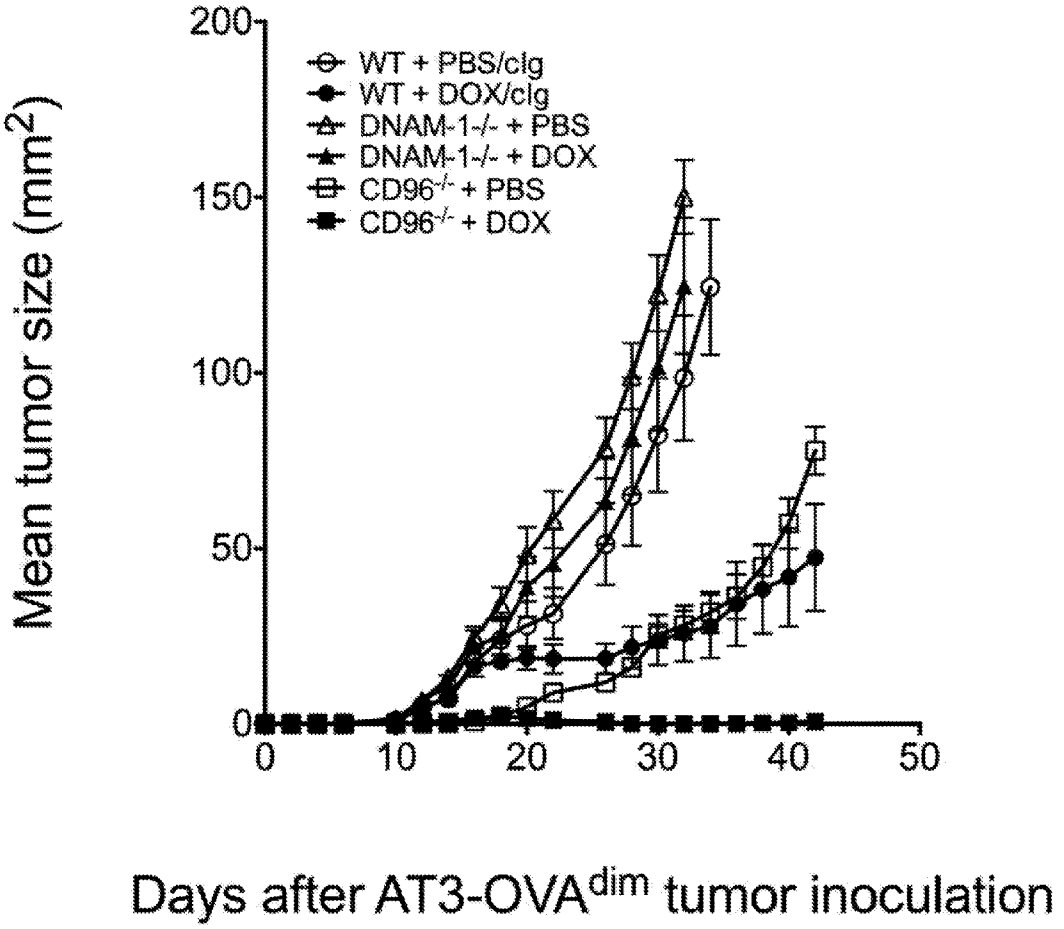

200
150
100
50
0
0
10
20
30
40
50
Mean tumor size (mm2)
WT + PBS/clg
WT + DOX/clg
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
Days after AT3-OVAdim tumor inoculation

Fig. 6

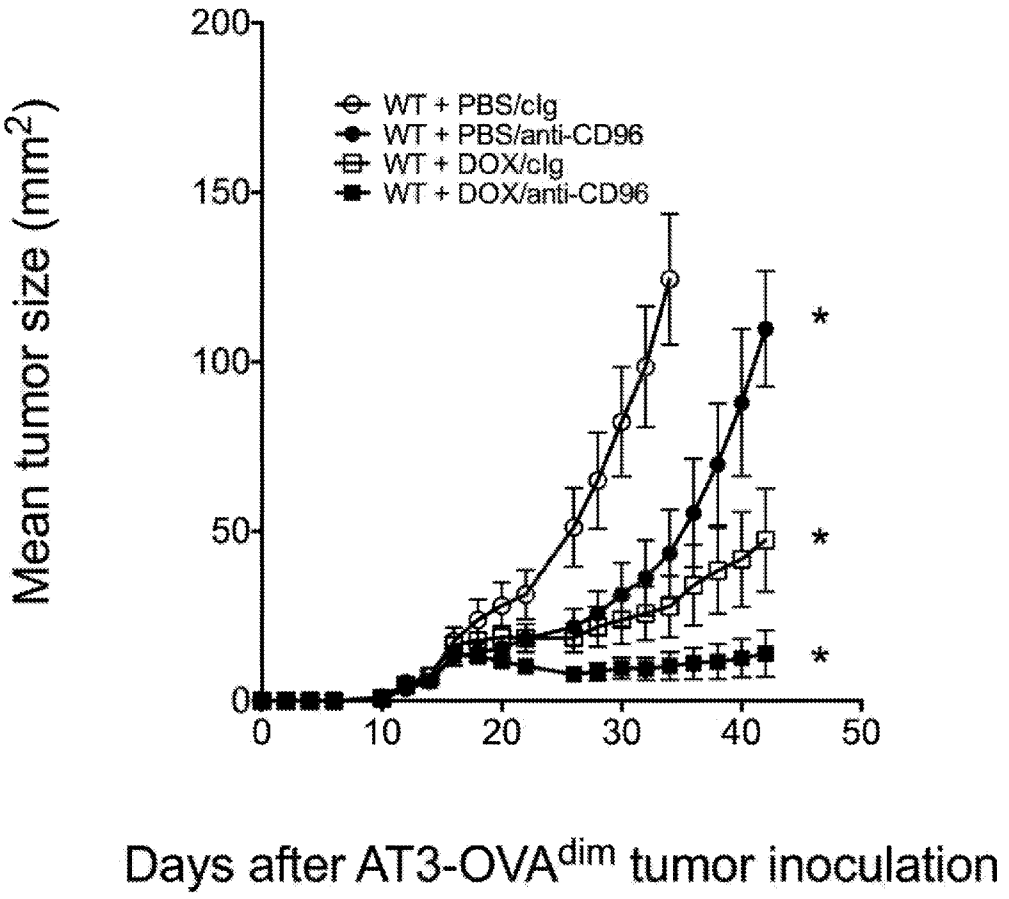

200
150
100
50
0
0
10
20
30
40
50
Mean tumor size (mm2)
WT + PBS/cIg
WT + PBS/anti-CD96
WT + DOX/cIg
WT + DOX/anti-CD96
*
*
*
Days after AT3-OVAdim tumor inoculation

Fig. 7

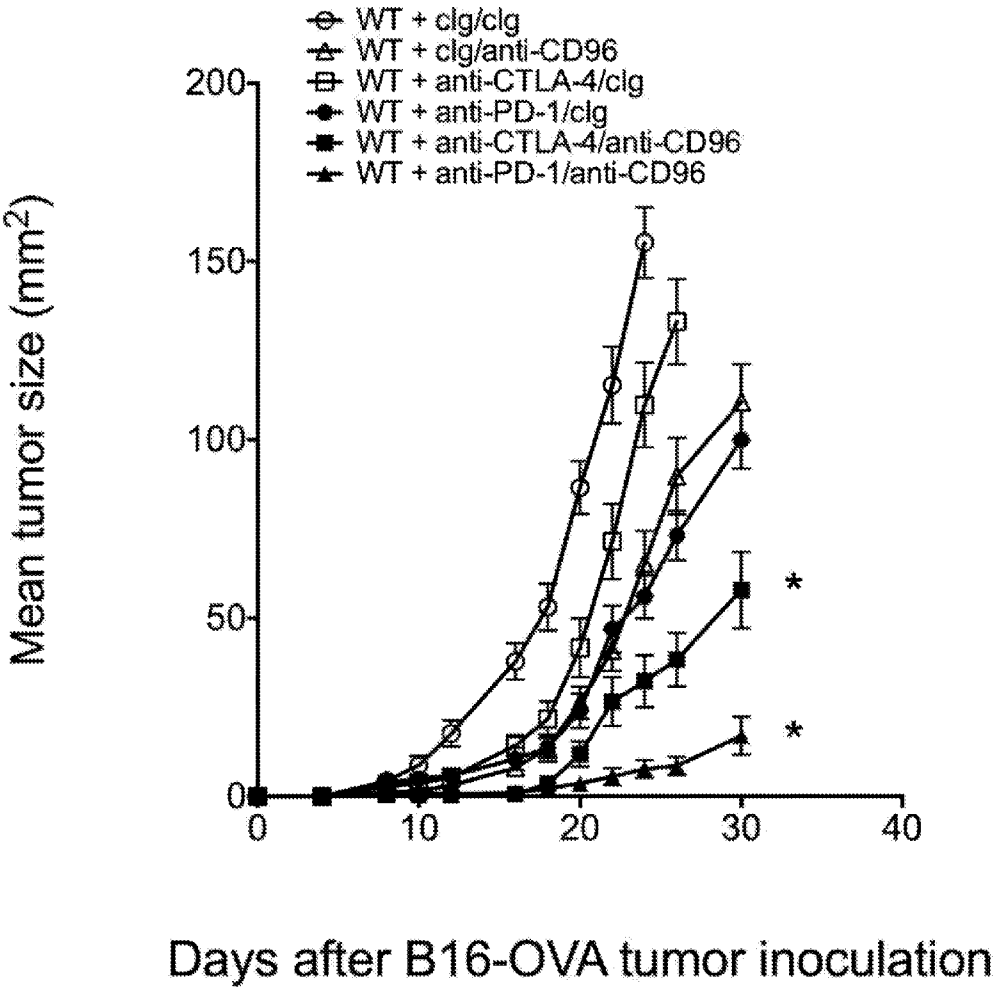

WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
*
*
Days after B16-OVA tumor inoculation

Fig. 8

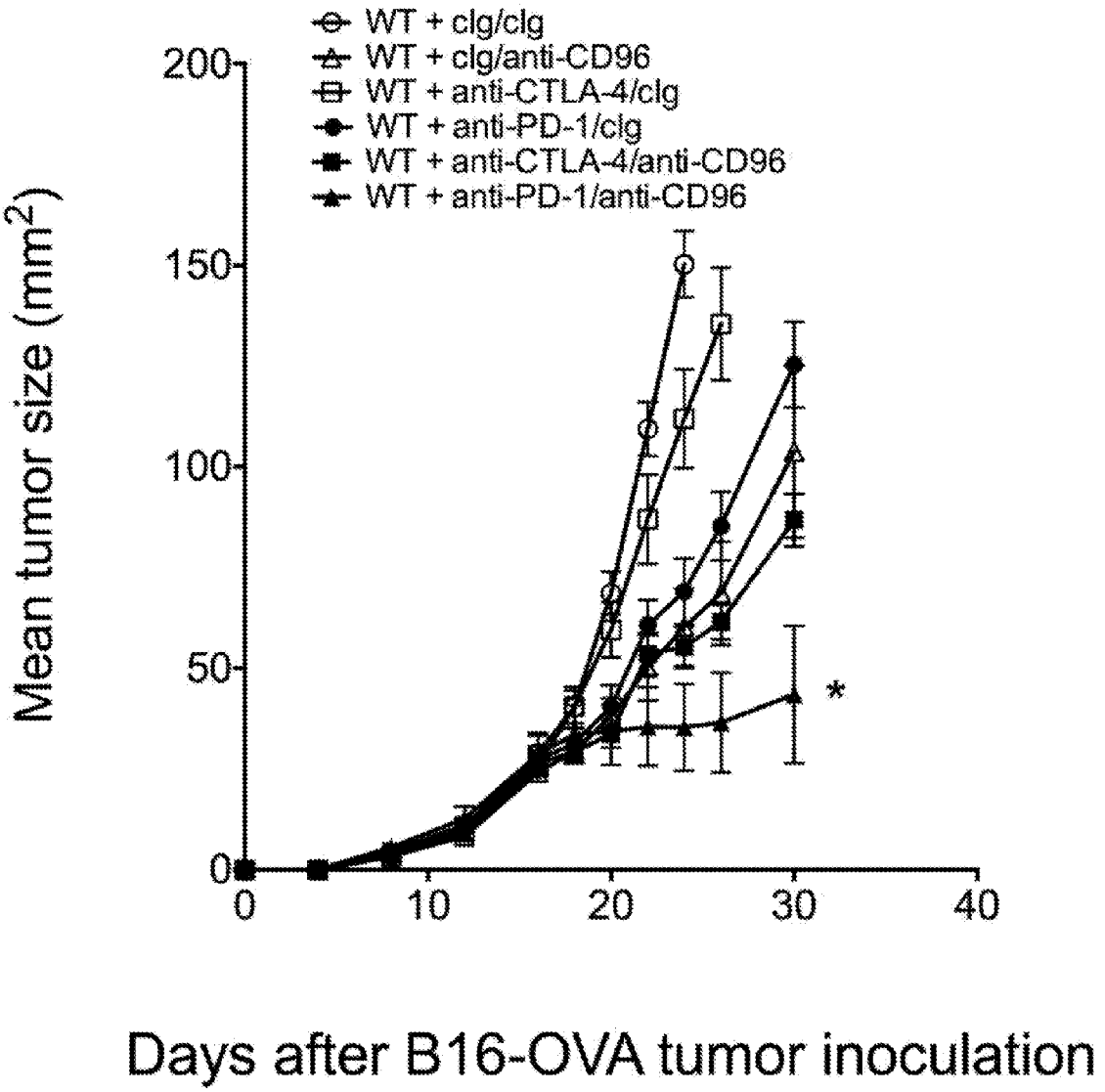

WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
*
Days after B16-OVA tumor inoculation

Fig. 9

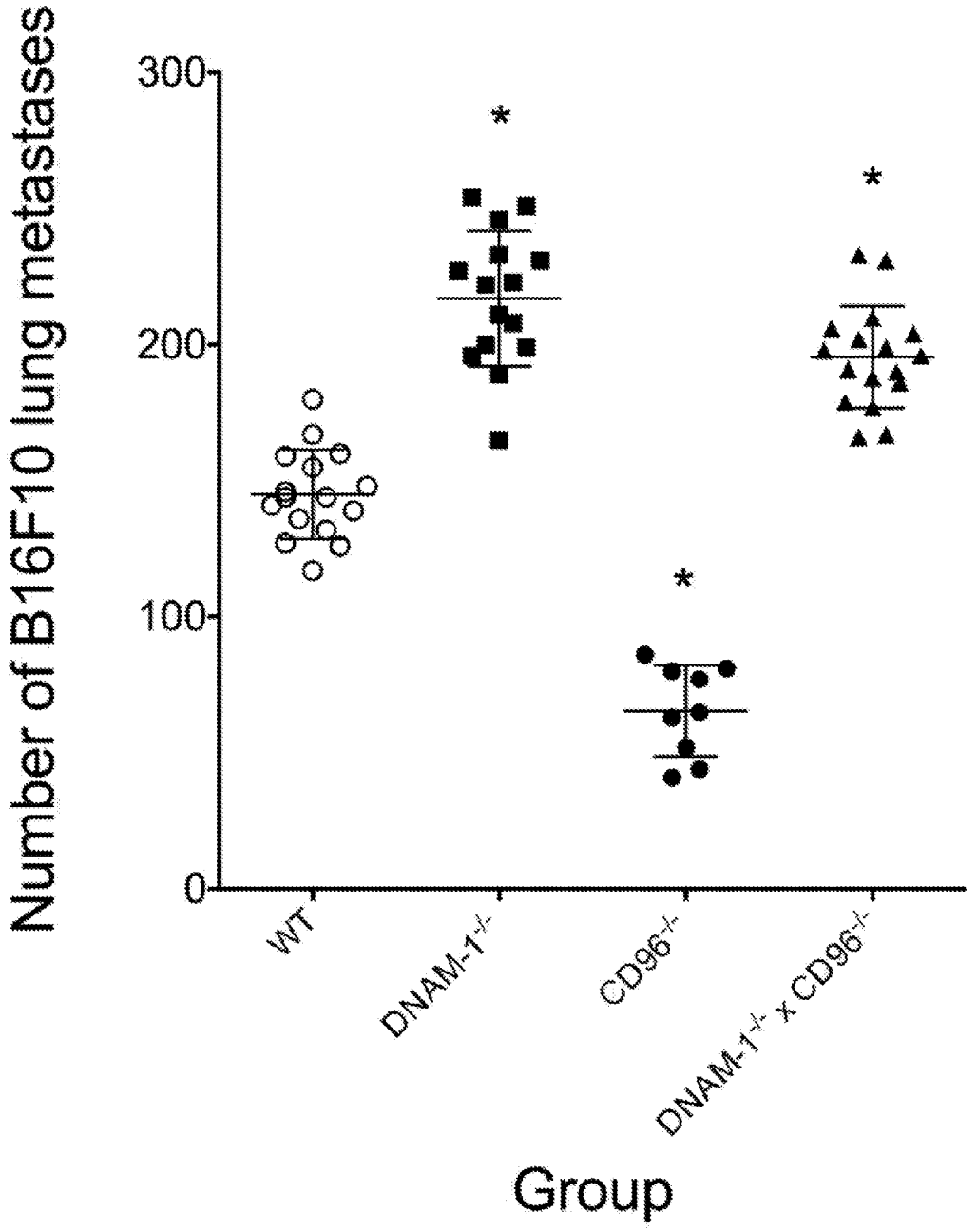

Number of B16F10 lung metastases
300
200
100
0
*
*
*
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 10

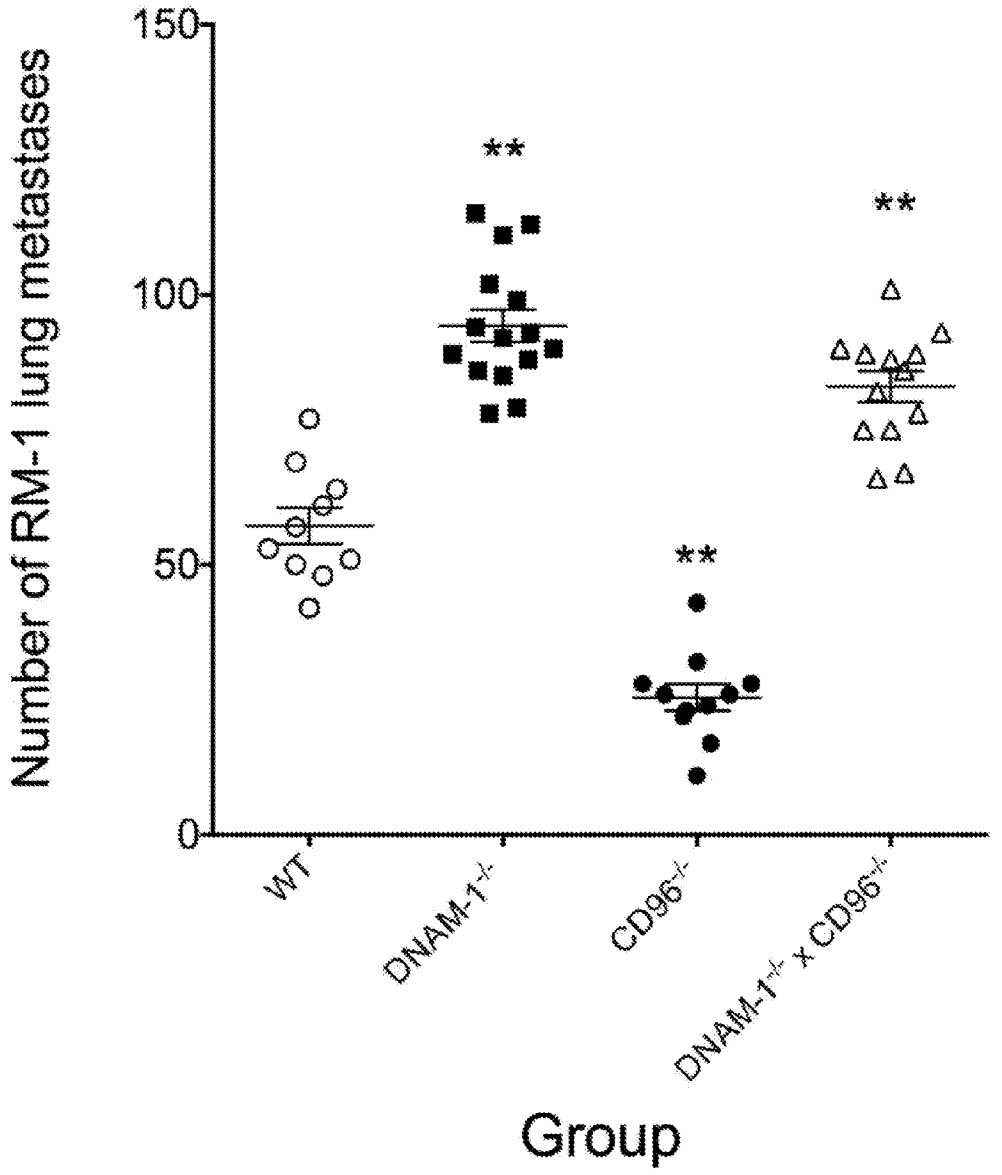

Number of RM-1 lung metastases
150
100
50
0
**
**
**
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 11

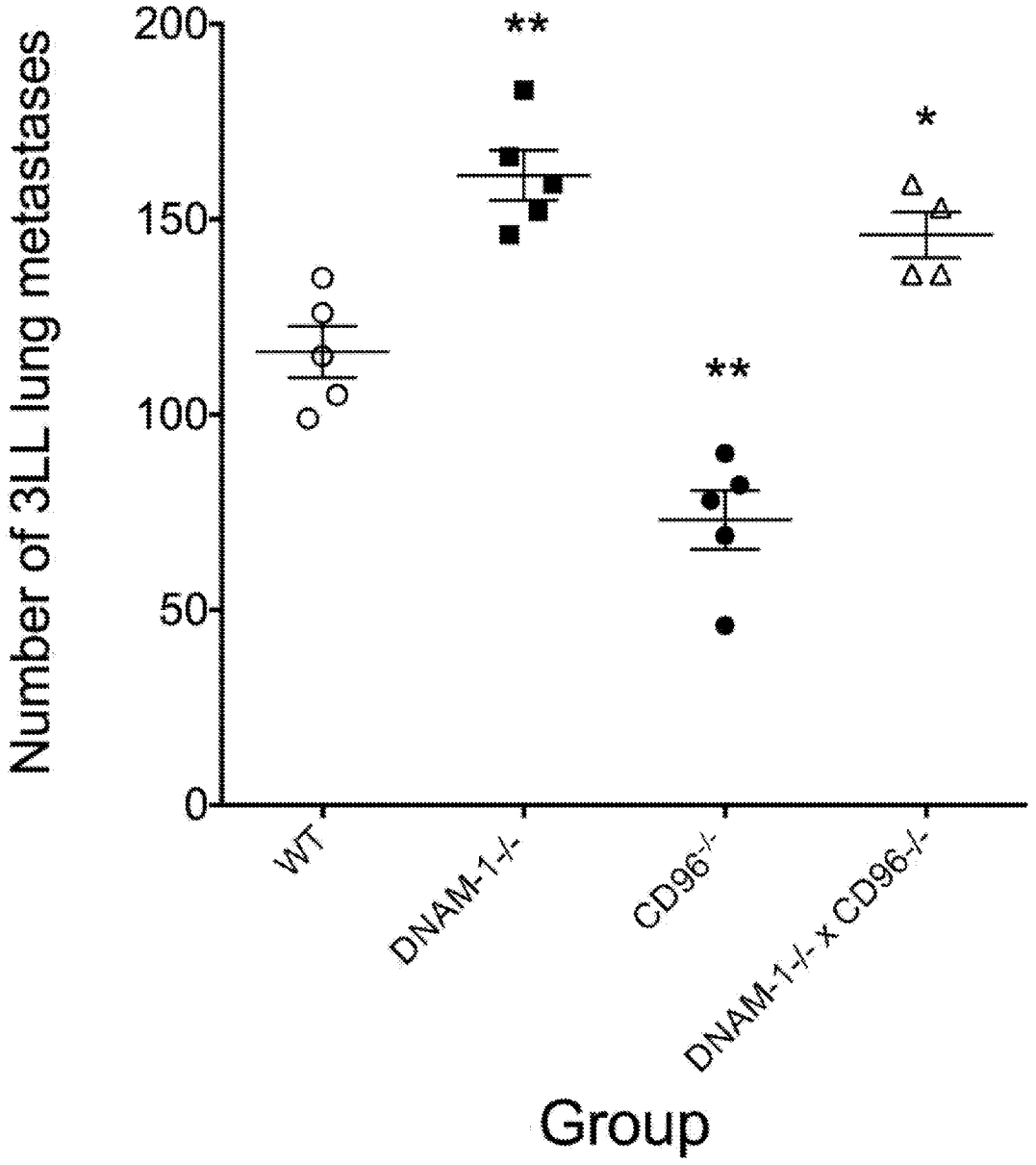

Number of 3LL lung metastases
200
150
100
50
0
**
**
*
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 12

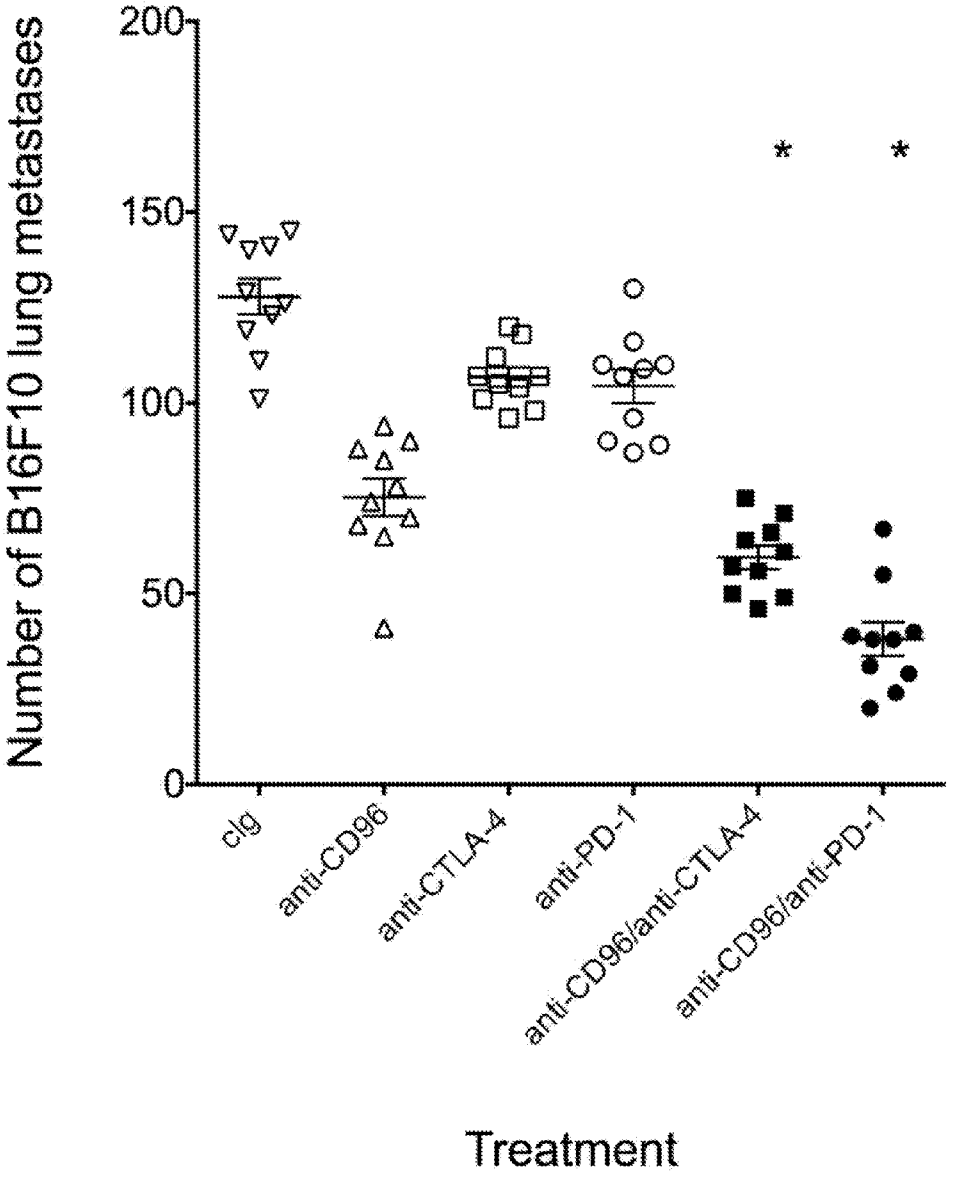

Number of B16F10 lung metastases
200
150
100
50
0
*
*
clg
anti-CD96
anti-CTLA-4
anti-PD-1
anti-CD96/anti-CTLA-4
anti-CD96/anti-PD-1
Treatment

Fig. 13

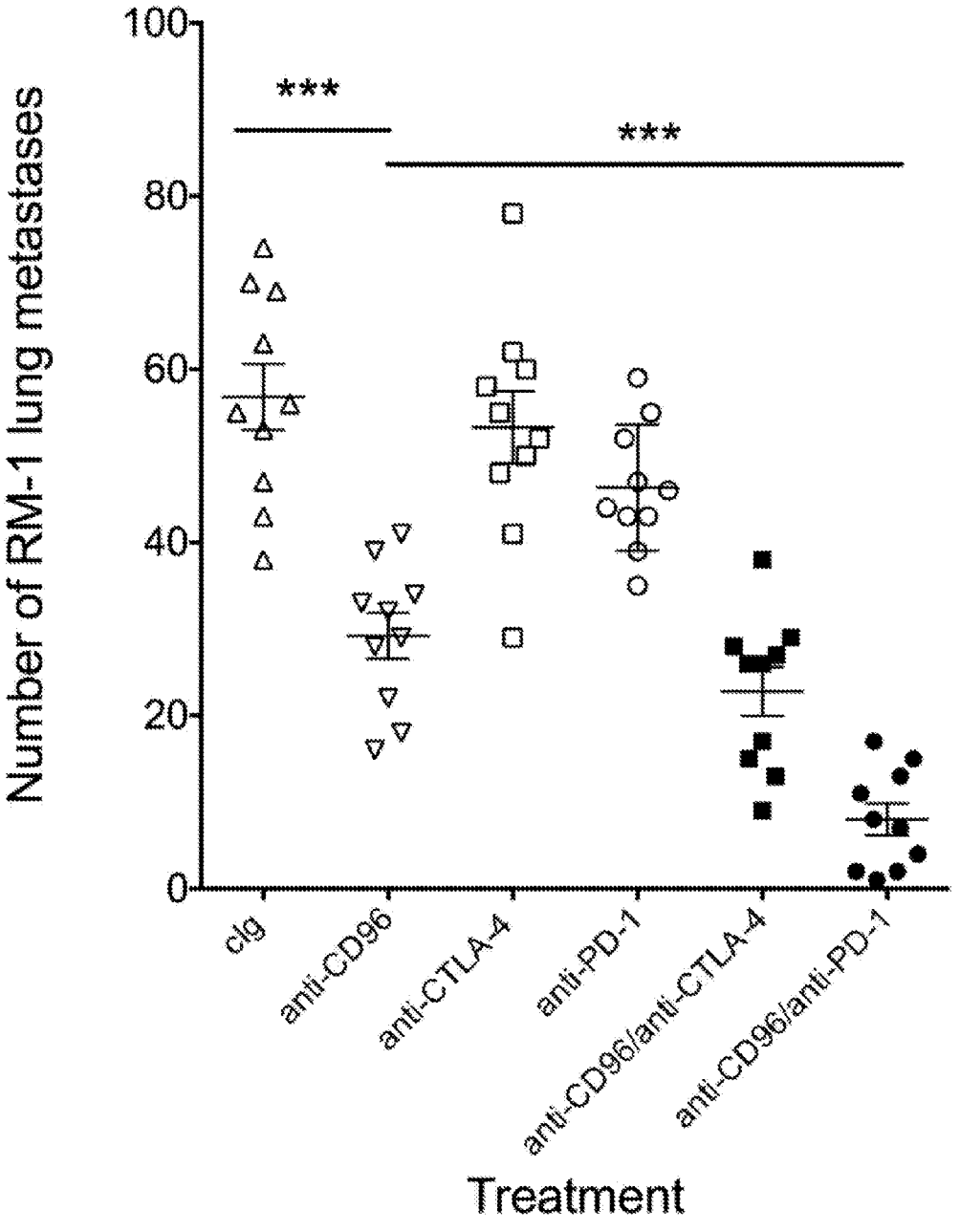

Number of RM-1 lung metastases
100
80
60
40
20
0
***
***
clg
anti-CD96
anti-CTLA-4
anti-PD-1
anti-CD96/anti-CTLA-4
anti-CD96/anti-PD-1
Treatment

Fig. 14

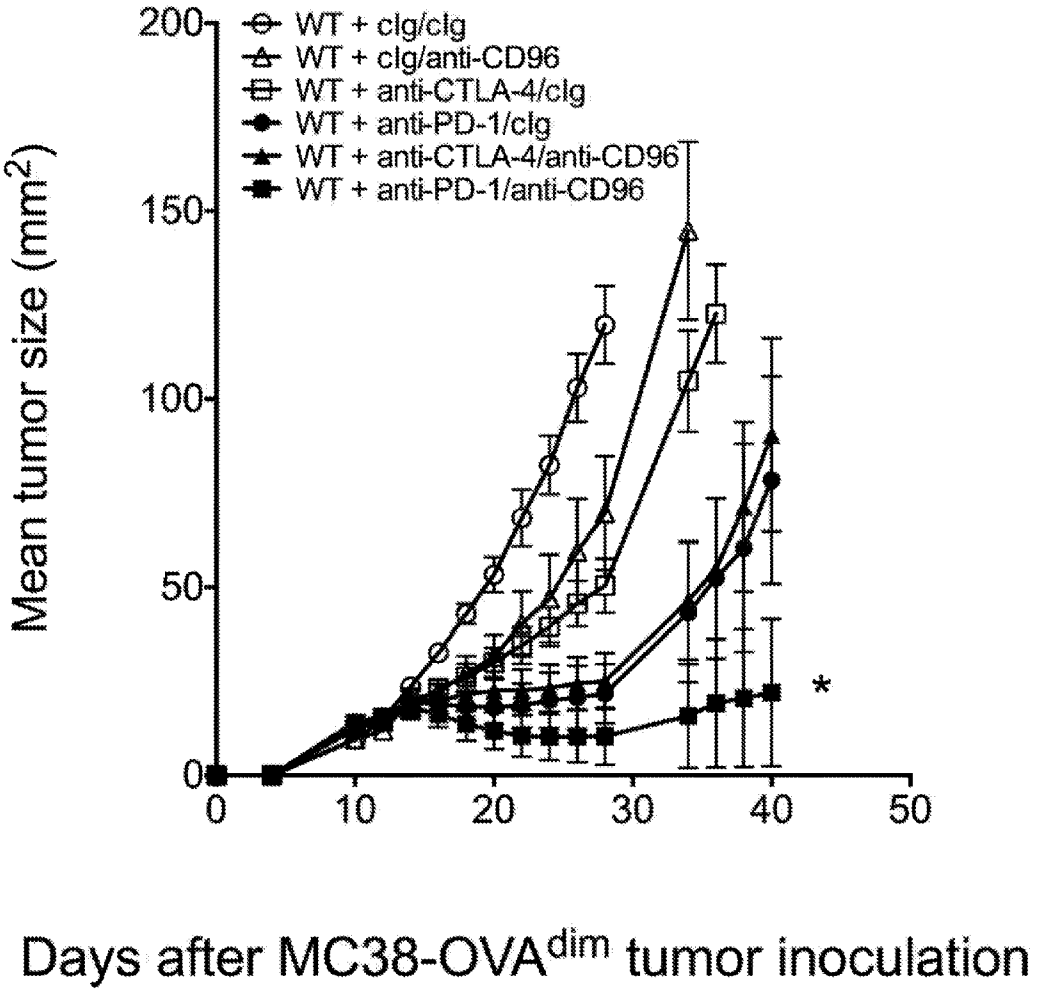
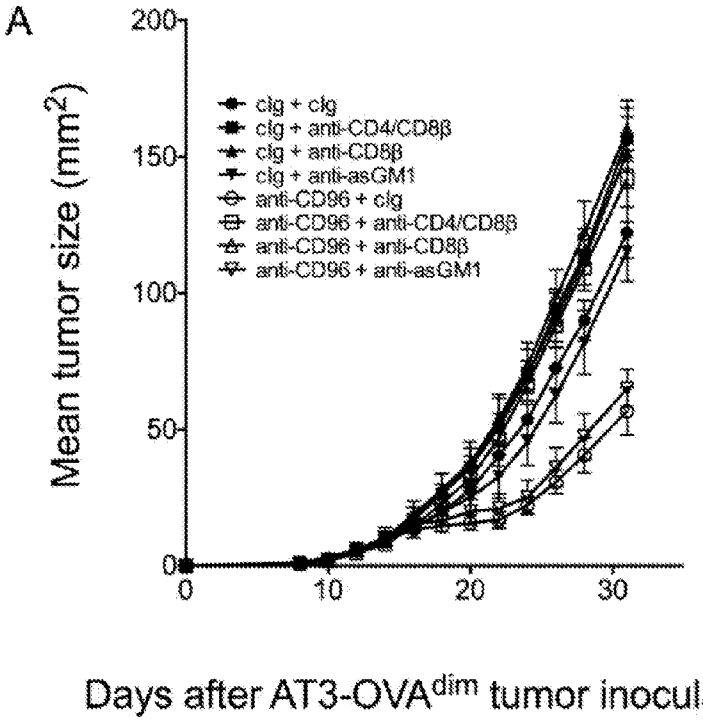

WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
*
Days after MC38-OVAdim tumor inoculation

Fig. 15


A
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
clg + clg
clg + anti-CD4/CD8β
clg + anti-CD8β
clg + anti-asGM1
anti-CD96 + clg
anti-CD96 + anti-CD4/CD8β
anti-CD96 + anti-CD8β
anti-CD96 + anti-asGM1
Days after AT3-OVAdim tumor inoculation

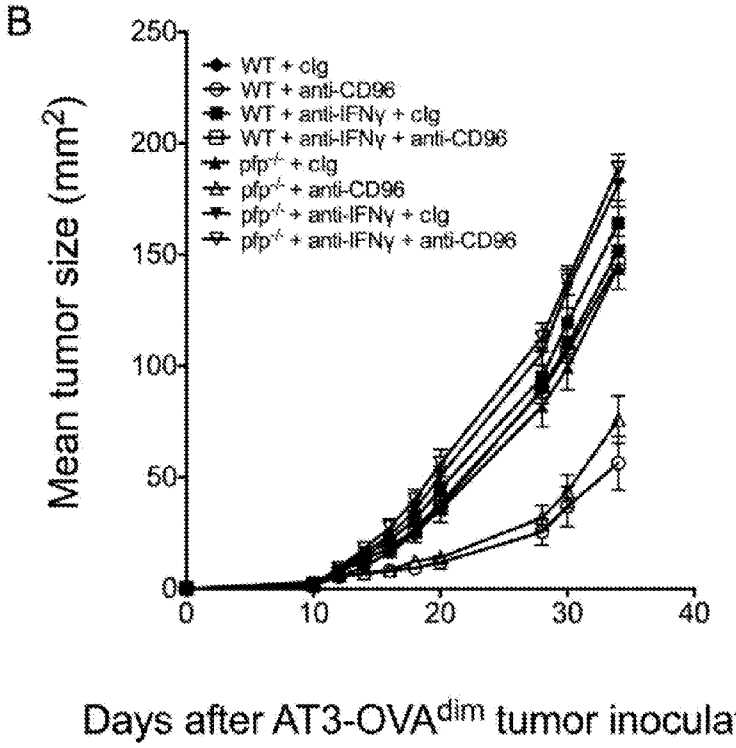

B
Mean tumor size (mm2)
250
200
150
100
50
0
0
10
20
30
40
WT + clg
WT + anti-CD96
WT + anti-IFNγ + clg
WT + anti-IFNγ + anti-CD96
pfp-/- + clg
pfp-/- + anti-CD96
pfp-/- + anti-IFNγ + clg
pfp-/- + anti-IFNγ + anti-CD96
Days after AT3-OVAdim tumor inoculation

Fig. 16

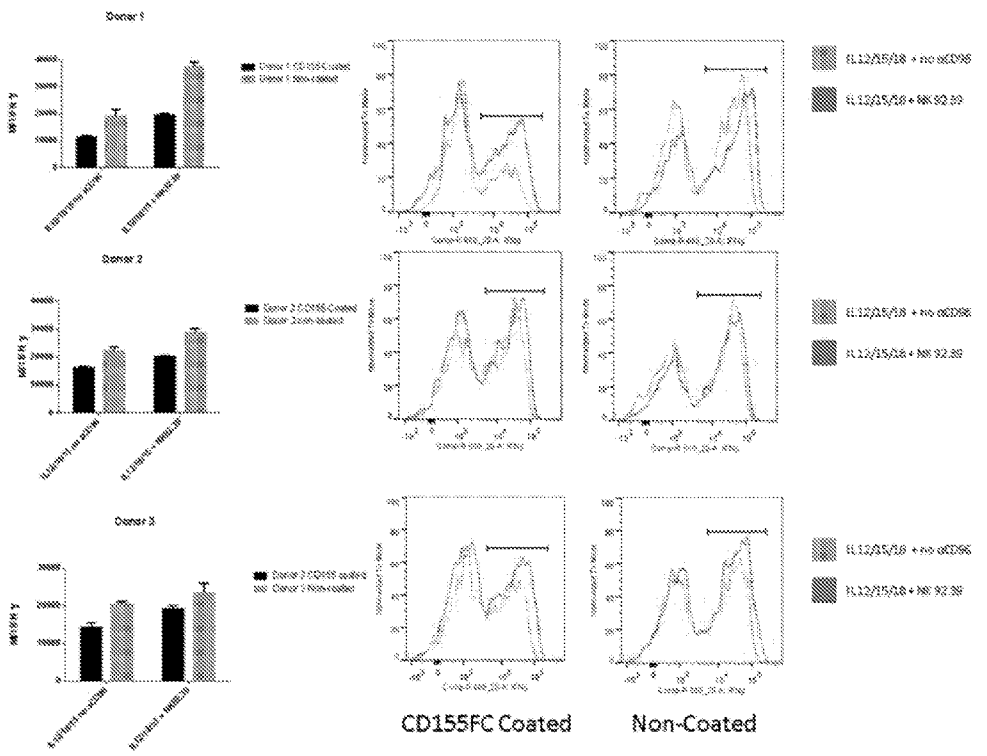


FIG. 17A

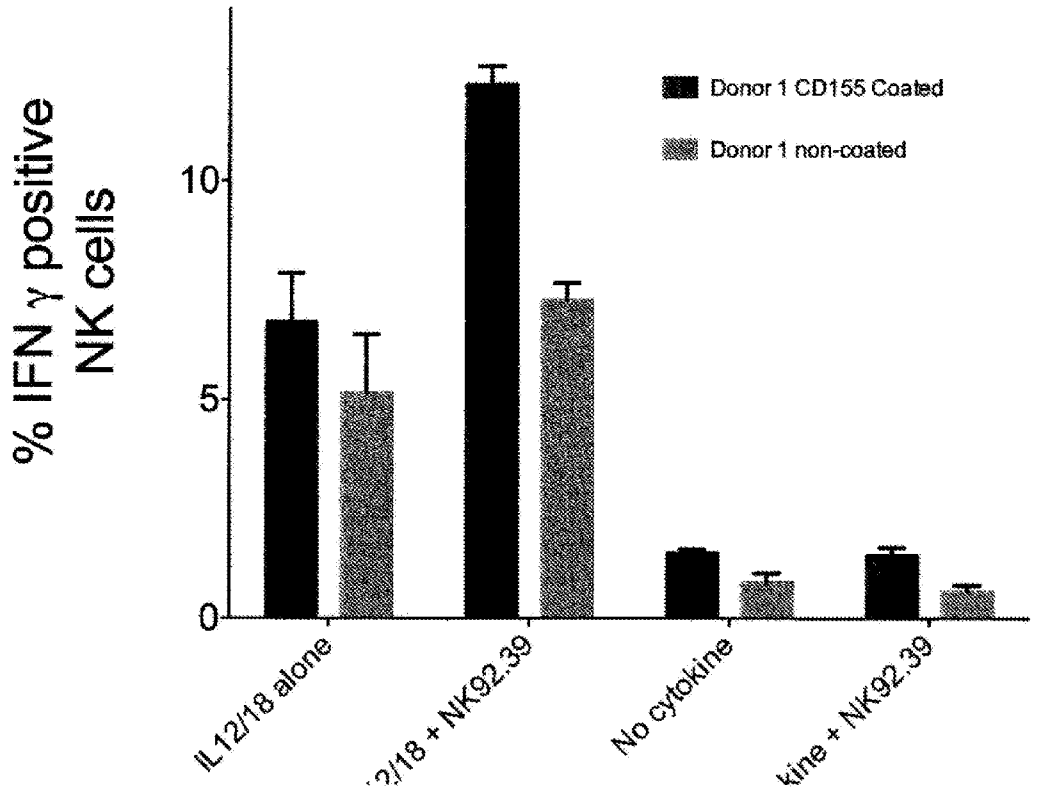
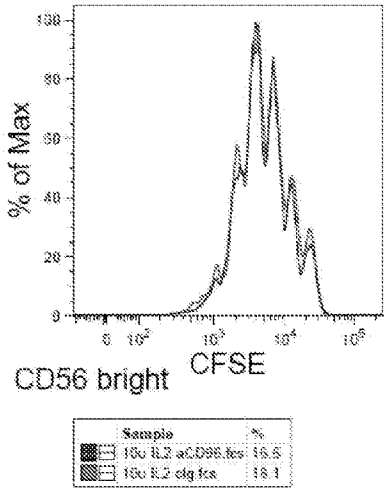
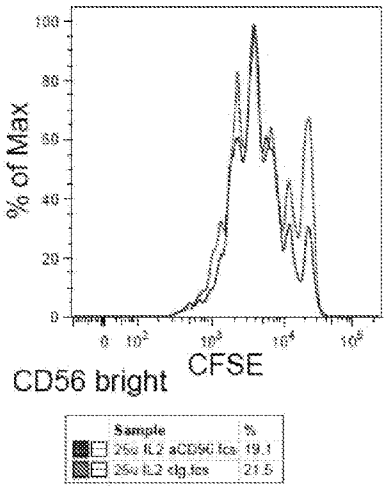

% IFN γ positive NK cells
10
5
0
Donor 1 CD155 Coated
Donor 1 non-coated
IL12/18 alone
No cytokine

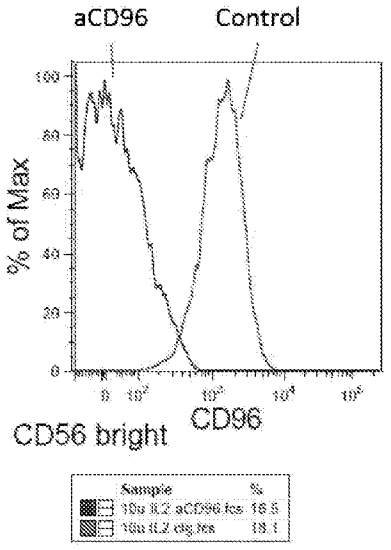
FIG. 17B



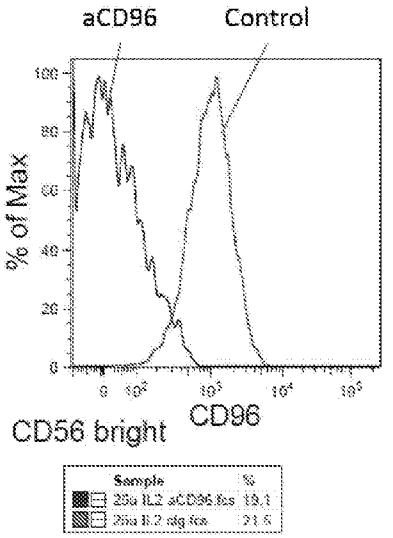
10unit/ml
IL-2



25unit/ml
IL-2



10unit/ml
IL-2



25unit/ml
IL-2

Fig. 18

IMMUNORECEPTOR MODULATION FOR TREATING CANCER AND VIRAL INFECTIONS

TECHNICAL FIELD

[0001] THIS INVENTION relates to the immunoreceptor CD96. More particularly, this invention relates to inhibition of CD96 to thereby enhance the ability of the immune system to target tumours and other diseases or conditions that can evade the immune system.

BACKGROUND

[0002] The progression of a productive immune response requires that a number of immunological checkpoints be passed. Passage may require the presence of excitatory co-stimulatory signals or the avoidance of negative or co-inhibitory signals, which act to dampen or terminate immune activity. The immunoglobulin superfamily occupies a central importance in this coordination of immune responses, and the CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4):B7.1/B7.2 receptor/ligand grouping represents the archetypal example of these immune regulators. In part the role of these checkpoints is to guard against the possibility of unwanted and harmful self-directed activities. While this is a necessary function, aiding in the prevention of autoimmunity, it may act as a barrier to successful immunotherapies aimed at targeting malignant self-cells that largely display the same array of surface molecules as the cells from which they derive. Therapies aimed at overcoming these mechanisms of peripheral tolerance, in particular by blocking the inhibitory checkpoints on T cells, offer the potential to generate antitumor activity, either as monotherapies or in synergism with other therapies that directly or indirectly enhance presentation of tumor epitopes to the immune system. Such anti-T cell checkpoint antibodies are showing promise in early clinical trials of advanced human cancers.

[0003] Furthermore, natural killer (NK) cells are innate lymphocytes critical to limit early tumor growth and metastasis[1]. NK cell functions are also regulated by the integration of signals transmitted by a wide range of activating and inhibitory receptors[2]. For example, the recognition of pathogen-derived or stress-induced ligands by activating receptors such as NCRs, NKG2D, or DNAM-1 stimulate NK cells cytotoxicity and the secretion of pro-inflammatory mediators such as interferon gamma (IFN-γ)[3]. In contrast, inhibitory receptors protect target cells from NK cell-mediated killing[4]. These receptors mostly recognize MHC class I and MHC class I-related molecules and include the KIR (killer cell immunoglobulin-like receptors) and LIR (leukocyte immunoglobulin-like receptors) families, the Ly49 family in mice and the CD94/NKG2 heterodimers in both species.

[0004] An emerging group of immunoglobulin superfamily members that interact with ligands of the nectin and nectin-like (necl) family has recently been described to influence NK cell and T cell functions[5]. These include CD226 (DNAM-1)[6], CD96 (TACTILE)[7], TIGIT (T cell immunoglobulin and ITIM domain)[8,9], and CRTAM (class I restricted T cell-associated molecule)[10]. DNAM-1 and TIGIT are the most extensively studied members of this family and they share a common ligand, CD155 (necl-5; PVR) and CD112 (nectin-2; PVRL2)[8,11]. TIGIT also bind an additional ligand CD113 (PVRL3)[8]. The functions of DNAM-1 and TIGIT on NK cells are reportedly counter-balancing[12]. In vitro, DNAM-1 potentiates the cytotoxicity of NK cells against a wide range of tumor cells[13,14] and is critical for tumor immunosurveillance in vivo[13,15,16]. In contrast, TIGIT bear an ITIM motif and has been proposed prevent self-tissue damage similar to inhibitory Ly49 or KIR interactions with MHC class I[17]. Indeed, engagement of TIGIT by CD155 has been shown to limit IFNγ production and cytotoxicity by NK cells in vitro[18,19]. However, the role of TIGIT in NK cell biology relative to the other nectin receptors DNAM-1 and CD96 remains to be assessed in vivo.

[0005] Despite being cloned 20 years ago[7], little is known about CD96, the other Ig family member that shares CD155 ligand with DNAM-1 and TIGIT[20,21]. In humans, CD96 expression is largely confined to NK cells, CD8 T cells, and CD4 T cells 7. The major ligand of CD96 is CD155, but CD96 has also been reported to associate with CD111 (nectin-1) and play a role in promoting NK and T cell adhesion[21,22].

SUMMARY

[0006] Surprisingly, the present inventors have discovered that CD96 acts as a negative regulator of T cell and NK cell anti-tumor functions. Accordingly, the invention is broadly directed to use of agents that at least partly block or inhibit CD96 to thereby reduce or relieve CD96-mediated immune inhibition to enhance or restore immune surveillance in the mammal. In certain embodiments, this may facilitate treatment of diseases or conditions responsive at least partial blocking or inhibition of CD96, such as cancers and/or viral infections.

[0007] In a first aspect, the invention provides a method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

[0008] Suitably, the step of inhibiting or reducing CD96 activity in the mammal does not include, or at least depend upon, killing of CD96-expressing cells in the mammal. In some embodiments, the step of inhibiting or reducing CD96 activity in the mammal includes inhibiting or reducing CD96 binding to CD155 and/or intracellular signaling in one or more cells of the mammal that express CD96. In some embodiments, the step of inhibiting or reducing CD96 activity in the mammal includes removing and/or down-regulating cell surface expression of CD96.

[0009] In one particular embodiment, the step of inhibiting or reducing CD96 activity in the mammal includes increasing or enhancing expression, production and/or secretion of one or more cytokines or chemokines. Preferably, the cytokine is interferon γ (IFN-γ). Typically, the one or more cells of the mammal are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδT cells, NKT cells, and natural killer (NK) cells.

[0010] In a preferred embodiment, the method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent cancer or cancer metastasis in the mammal.

[0011] In other embodiments, the method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent a viral infection in the mammal.

[0012] In a second aspect, the invention provides a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at

least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

[0013] In a third aspect, the invention provides a CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the method of the second aspect.

[0014] In one embodiment, the CD96-inhibitory agent is an antibody or antibody fragment.

[0015] In one particular embodiment, the CD96-inhibitory agent is an anti-cancer agent.

[0016] In another particular embodiment, the CD96-inhibitory agent is an anti-viral agent.

[0017] In a fourth aspect, the invention provides a CD96-inhibitory agent according to the third aspect for use according to the method of the first aspect.

[0018] Suitably, according to the aforementioned aspects the mammal is a human.

[0019] Unless the context requires otherwise, the terms "comprise", "comprises" and "comprising", or similar terms are intended to mean a non-exclusive inclusion, such that a recited list of elements or features does not include those stated or listed elements solely, but may include other elements or features that are not listed or stated.

[0020] The indefinite articles 'a' and 'an' are used here to refer to or encompass singular or plural elements or features and should not be taken as meaning or defining "one" or a "single" element or feature.

BRIEF DESCRIPTION OF THE FIGURES

[0021] FIG. **1**: CD96 competes with DNAM-1 for CD155 binding a, b The expression of CD96 was analyzed by flow cytometry on the indicated spleen lymphocyte populations from C57BL/6 WT (light grey) and CD96-mice (dark grey). The representative FACS Histograms (a) and the mean±SD (b) of 3 mice from one representative experiment out of 3 are shown. c, d The expression of CD96. DNAM-1 and TIGIT was determined on WT spleen NK cells freshly isolated or activated for 48 hrs with IL-2 (1000 U/ml). e. The binding of mouse CD155-Fc coupled with AF-647 to purified NK cells freshly isolated from WT, $CD96^{-/-}$, $DNAM\text{-}1^{-/-}$ or $DNAM\text{-}1^{-/-}$ $CD96^{-/-}$ mice was assessed at the indicated concentrations by flow cytometry. f. The binding of CD155-Fc coupled with AF-647 (10 μg/ml) was analyzed on purified WT NK cells in the presence of anti-CD96 and or anti-DNAM-1 mAbs. g. The binding of DNAM-1-Fc labeled with AF-647 (0.5-10 μg/ml) at the cell surface of BMDC was analyzed in the presence of 50 μg/ml of control Ig, recombinant CD96 or TIGIT-Fc. c-g. The representative FACS Histograms and the mean±SD of triplicate wells from one representative experiment out of at least 3 experiments are shown. ***$p<0.001$ Student T test.

[0022] FIG. **2**: CD96 engagement by CD155 regulate NK cell production of IFNγ. CD96 binding to CD155-Fc limits the production of IFN-γ by NK cells induced by exogenous cytokines (a, b, d) and NK cell receptors (c). a, b, d. We analyzed the intracellular production of IFN-γ by freshly purified $CD96^{-/-}$, $TIGIT^{-/-}$ and WT NK cells in the presence or absence of anti-CD96 (50 μg/ml) in response to IL-12 (25-100 μg/ml) and IL-18 (50 ng/ml) using plates coated with or without CD155-Fe (0.5 μg/well). c. We analyzed the intracellular production of IFN-γ by IL-2-activated NK cells from $CD96^{-/-}$ and WT mice using plates coated with anti-NK1.1 (2.5 μg/well) and CD155-Fc (0.5 μg/well). The representative FACS Histograms (a) and the mean±SD of triplicate wells (b, c, d) from one representative experiment out of 3 are shown. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Student T test.

[0023] FIG. **3**: CD96 limits NK cell-dependent tumor immunosurveillance. a, b. CD96 and DNAM-1 have an opposite role in the control of B16F10 metastasis. a. 2×10^5 B16F10 cells were intravenously injected into WT, $CD96^{-/-}$, $DNAM\text{-}1^{-/-}$ and $DNAM\text{-}1^{-/-}CD96^{-/-}$ mice and metastatic burden was quantified in the lungs after 14 days. Representative experiment out of 3. b. Pictures showing the lung of WT and $CD96^{-/-}$ mice two weeks after the injection of 2×10^5 and 5×10^5 B16F10 cells. Representative experiment out of two. c. CD96 and TIGIT compete with DNAM-1 for the binding of CD155 at the cell surface of B16F10. The binding of DNAM-1-Fc labeled with AF-647 (0.5-20 μg/ml) at the cell surface of B16F10 cells was analyzed in the presence of 50 μg/ml of control Ig, recombinant CD96 or TIGIT-Fc. The FACS histograms and the mean±SD of triplicate wells from one representative experiment out of 3 are shown. d. A 4 hr ^{51}Cr release assay was performed between B16F10 cells and IL-2-activated NK cells from WT, $DNAM\text{-}1^{-/-}$ and $CD96^{-/-}$ mice at the indicated effector target ratios. Solid circles represent WT NK cells, open circles represent $CD96^{-/-}$ NK cells and solid squares represents $DNAM\text{-}1^{-/-}$ NK cells. e-h. CD96 and DNAM-1 have an opposite role in the immunosurveillance of MCA induced fibrosarcoma mediated by NK cells. e-h Groups of 15-30 male, WT, $DNAM\text{-}1^{-/-}$ and $CD96^{-/-}$ and $DNAM\text{-}1^{-/-}CD96^{-/-}$ mice were injected with MCA (100 μg/mouse). The survival (e-g) and the growth curves of individual mice with sarcoma (h) are shown. f. WT mice were treated with an anti-CD96, anti-DNAM-1 or anti-CD155 mAbs as defined in the Materials and Methods. g. WT and $CD96^{-/-}$ mice were injected with 100 μg MCA and treated with either a control antibody, anti-IFN-γ antibody, or anti-asialoGML. *$p<0.05$ Mantel-Cox test.

[0024] FIG. **4**: Anti-CD96 mAb has single agent activity and enhances the anti-tumor responses of anti-PD1. C57BL/6 wild type (WT) mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16, 20 and 24 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p) or anti-PD-1 (RMP1-14, 250 μg i.p.). Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared to cIg alone by Mann-Whitney test).

[0025] FIG. **5**: Anti-CD96 mAb enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy. C57BL6 wild type (WT), $DNAM\text{-}1^{-/-}$, and $CD96^{-/-}$ mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 14 with control PBS or DOX (50 microliters, 2 mM. intratumor). Some groups of WT mice also received on day 12, 14, 18, 21, 24 and 28 intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p) or anti-DNAM-1 (480.1, 250 μg i.p.). Means±SEM of 5 mice per group (mm^2) are shown.

[0026] FIG. **6**: Enhanced anti-tumor responses of Doxorubicin (DOX) chemotherapy with host CD96 deficiency. C57BL/6 wild type (WT), $DNAM\text{-}1^{-/-}$. and $CD96^{-/-}$ mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16 with control PBS or DOX (50 microliters, 2 mM, intratumor). Means±standard errors of 5 mice per group (mm^2) are shown.

[0027] FIG. **7**: Anti-CD96 mAb enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy. C57BL6 wild type (WT) mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16 with control PBS or DOX (50 microliters, 2 mM, intratu-

mor). Some groups of WT mice also received on day 16, 20, and 23 intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p). Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared to cIg alone by Mann-Whitney test).

[0028] FIG. **8**: Early anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs. C57BL/6 wild-type (WT) mice were injected subcutaneously with B16-OVA melanoma cells (10^5 cells) and treated on day 1, 5, and 9 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone, by Mann-Whitney test).

[0029] FIG. **9**: Late anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 mAb. C57BL/6 wild-type (WT) mice were injected subcutaneously with B16-OVA melanoma cells (10^5 cells) and treated on day 16, 20, and 24 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

[0030] FIG. **10**: Host CD96 promotes B16F10 lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenously with B16F10 melanoma cells (10^5 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means±SEM of 9-17 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

[0031] FIG. **11**: Host CD96 promotes RM-1 lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenous with RM1 prostate carcinoma cells (10^4 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means±SEM of 10-15 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

[0032] FIG. **12**: Host CD96 promotes 3LL lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenously with 3LL lung carcinoma cells (10^5 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means±SEM of 5 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

[0033] FIG. **13**: Anti-CD96 suppresses B16F10 lung metastasis, alone and in combination with T cell checkpoint blockade. C57BL/6 wild type (WT) mice were injected intravenous with B16F10 melanoma cells (10^5 cells). On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means±SEM of 5 mice per group are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

[0034] FIG. **14**: Anti-CD96 suppresses RM-1 lung metastasis, alone and in combination with T cell checkpoint blockade. C57BL/6 wild type (WT) mice were injected intravenous with RM-1 prostate carcinoma cells (10^4 cells). On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means±SEM of 5 mice per group are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

[0035] FIG. **15**: Late anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 mAb. C57BL/6 wild-type (WT) mice were injected subcutaneously with MC38-OVA^{dim} colon adenocarcinoma cells (10^6 cells) and treated on day 14, 18, 22, and 26 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

[0036] FIG. **16**. Mechanism of the anti-tumor effect of anti-CD96 alone against AT3-OVA^{dim} mammary carcinoma. (A-B) C57BL/6 wild-type (WT) and $pfp^{-/-}$ mice were injected s.c. with AT3-OVA^{dim} mammary carcinoma (1×10^6 cells). On (A) day 16, 20, and 24 or (B) day 12, 16 and 18 after tumor inoculation mice were treated with i.p. injections of cIg (250 μg i.p.) or anti-CD96 mAb (250 μg i.p.). Some groups of mice were treated on (A) days 14, 16, and 23 after tumor inoculation with cIg, anti-CD4/anti-CD813 (100 gag i.p.) or anti-asGM1 (100 μg i.p.) or (B) days 10, 12 and 18 after tumor inoculation with cIg or anti-IFN-γ (250 μg i.p.). Mice were then monitored for tumor growth. Means±SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ of cIg-treated mice compared with anti-CD96 mAb-treated mice by Mann-Whitney test).

[0037] FIG. **17**. In Vitro NK cell activation. To analyse the production of IFNγ from human NK cells, 96 well U bottom plates were coated with recombinant human CD155-Fc chimera overnight at 4° C. (0.25 μg/well). After three washes, 2.5×10^4 human NK cells, freshly isolated from buffy coats and FACS sorted were plated in complete RPMI supplemented with human IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (100 ng/ml) in the presence or absence of human anti-CD96 antibody (clone NK92.39, 50 μg/ml). Cultures were also set up in wells not containing CD155-Fc coating. After 24 hours incubation, cells were harvested and analysed for IFNγ production by Flow Cytometry. All conditions were run in triplicate and error bars represent the ±SEM. (A) Flow cytometry results using IL-12, 18 and 15; and (B) result from a different donor (not donor 1 shown in 17A) showing the proportion of IFNγ positive NK cells.

[0038] FIG. **18**. Binding of human CD96 mAb (NK92.39) to human NK cells reduced the levels of CD96 present on the NK cell surface. Total NK cells were purified from peripheral blood mononuclear cells (PBMCs) by negative selection using human NK cell isolation kit (Miltenyi Biotec.). Isolated NK cells were then labeled with carboxyfluorescein diacetate succinmidyl ester (CFSE; Biolegend) to measurecellular pro-

liferation. CFSE-labeled NK cells were plated in 96 well U-bottom plate at 5×10^4 cells/well and stimulated with recombinant IL-2 at indicated concentrations (10 units/ml and 25 units/ml), in the presence of control IgG or anti-CD96 mAb (clone NK92-39) at 30 μg/ml. NK cells were assessed for changes in proliferation (A) or the presence/absence of surface CD96; and (B) at day 3 and 6 using BD FACS Canto 11 (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

DETAILED DESCRIPTION

[0039] The present invention is at least partly predicated on the unexpected discovery that CD96 is highly expressed by resting NK cells and T cell subsets and competes with DNAM-1 for the binding of CD155 on resting NK cells. Using $CD96^{-/-}$ mice, it is demonstrated that CD96 dampens or suppresses NK cell production of IFN-γ in vitro and in vivo, through competition with DNAM-1 for CD155 binding and also through a direct inhibition. Furthermore, $CD96^{-/-}$ mice were shown to be more resistant to 3'-methylcholanthrene (MCA)-induced tumor formation as an indicator of carcinogenesis, or B16F10 (melanoma), RM-1 (prostate cancer), 3LL (lung cancer) experimental metastasis. In human NK cells, administration of anti-CD96 antibody appears to remove or result in the loss of cell surface CD96 and/or cause a down-regulation of CD96 cell surface expression. Based on these observations, it is proposed that CD96 normally acts as a negative regulator of T and NK cell anti-tumor functions, particularly although not exclusively through suppression of IFN-γ production and/or secretion. Accordingly, the invention provides methods of relieving or reducing the negative immunoregulatory function of CD96 to thereby promote or restore immune surveillance, particularly by T cells and NK cells, to thereby treat or prevent cancer, cancer cell metastasis and/or viral infections.

[0040] An aspect of the invention therefore provides a method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

[0041] By "relieving immune inhibition" in the context of CD96 is meant at least partly eliminating, removing or overcoming a normal activity or function of CD96 in suppressing or inhibiting one or more immune functions of cells that normally express CD96. Typically, the one or more cells that normally express CD96 are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδT cells, NKT cells, and natural killer (NK) cells. In some embodiments, relieving immune inhibition may include or relate to abrogating peripheral tolerance to foreign pathogens, host cells displaying foreign pathogens (e.g displaying foreign pathogen-derived peptides in the context of self-MHC) and/or cancerous cells or tissues of the host.

[0042] By "enhance or restore immune surveillance" is meant at least partly improving or promoting the ability of one or more elements of the immune system to monitor, detect and/or respond to foreign pathogens, host cells displaying foreign pathogens (e.g displaying foreign pathogen-derived peptides in the context of self-MHC) and/or cancerous cells or tissues of the host. Suitably, the elements of the immune system are one or more cells that normally express CD96, such as T cells, inclusive of $CD4^+$ and $CD8^+$ T cells γδT cells, NKT cells and natural killer (NK) cells.

[0043] At least partly inhibiting or reducing CD96 activity in one or more cells of the mammal may be performed, facilitated or achieved by administration of a "CD96-inhibitory agent" to the mammal. A CD96-inhibitory agent may be any molecule that possesses or displays an ability to at least partly inhibit or reduce a biological activity of CD96. Biological activities of CD96 include one or more of binding CD155, cell surface expression, eliciting intracellular signaling and/or stimulating or inducing expression and/or secretion of cytokines or chemokines. Preferably, the cytokines or chemokines include any pro-inflammatory cytokine or chemokine inclusive of MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ, although without limitation thereto. Preferably, the cytokine is IFN-γ.

[0044] As disclosed herein, CD96 is a transmembrane protein which in the human, exists in two isoforms. Isoform 1 has been detected in acute myeloid leukaemia and includes additional amino acids compared with isoform 2. Isoform 2 is the more common form in humans and the predicted domain architecture of isoform 2 has three (3) external immunoglobulin-like domains (domains 1, 2 and 3) as listed in Table 1. The amino acid sequence of human CD96 isoform 1 is set forth in SEQ ID NO:2. The nucleotide sequence encoding isoform 2 is set forth in SEQ ID NO:1 Murine CD96 exists as a single isoform, the amino acid sequence of which is set forth in SEQ ID NO:4. The nucleotide sequence encoding murine CD96 is set forth in SEQ ID NO:3 The external immunoglobulin-like domains (domains 1, 2 and 3) of murine CD96 are also listed in Table 1.

[0045] In a preferred form, the CD96-inhibitory agent binds or interacts with an amino acid sequence of one or a plurality of external immunoglobulin-like domains of CD96. For example, the CD96-inhibitory agent may bind or interact with an amino acid sequence of: domain 1; domain 2; domain 3; domain 1 and domain 2; domain 1 and domain 3: domain 2 and domain 3; or domain 1, domain 2 and domain 3.

[0046] In one embodiment, the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of human CD96 isoform 2.

[0047] It will also be appreciated that the CD96-inhibitory agent may bind or interact with other CD96 domains or amino acid sequences in addition to one or a plurality of the external or extracellular immunoglobulin-like domains.

[0048] In one embodiment, the CD96-inhibitory agent inhibits, blocks or antagonizes a binding interaction between CD96 and CD155. By way of example only, the CD96-inhibitory agent may bind to an extracellular domain of CD96, or a portion thereof, that is capable of interacting with CD155 (e.g. binding CD155 or being bound by CD155) to thereby at least partly inhibit or block CD96 binding to CD155.

[0049] In another embodiment, the CD96-inhibitory agent is a molecule that possesses or displays an ability to inhibit or reduce CD96 signaling activity. Inhibition or reduction of CD96 signaling activity may be through inhibiting, blocking or antagonizing a binding interaction with CD155 or may be through blocking CD96-initiated signaling that would normally occur in response to CD155 binding. By way of example, CD96 comprises an immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs are structurally defined as 6-amino acid sequences comprising a tyrosine (Y) residue with partly conserved N-terminal (Y-2) and C-terminal (Y+3) residues. A general but non-limiting motif is (S/I/V/LX-

YXXI/V/L), wherein X is any amino acid. For example, isoform 1 of CD96 comprises the ITIM sequence IKYTCI wherein Y is residue 566.

[0050] It has been proposed that when co-aggregated with activating receptors, ITIMs are phosphorylated by Src-family tyrosine kinases, which enables them to recruit Src homology 2 domain-containing phosphatases (PTPases) that antagonize activation signals. Accordingly, in one embodiment the CD96-inhibitory agent possesses or displays an ability to inhibit or reduce CD96 signaling activity mediated by the CD96 ITIM. Preferably, inhibition or reduction of CD96 signaling activity mediated by the CD96 ITIM enables increased or enhanced cytokine (e.g IFN-γ) expression, production and/or secretion by a cell expressing CD96.

[0051] In another embodiment, the CD96-inhibitory agent is a molecule that removes cell surface CD96 and/or reduces or down-regulates cell surface expression of CD96.

[0052] The CD96-inhibitory agent may be a protein (inclusive of peptides, antibodies and antibody fragments), a nucleic acid (inclusive of inhibitory RNA molecules such as ribozymes, RNAi, miRNA and siRNA, although without limitation thereto), a lipid, a carbohydrate, a small organic molecule or any combination of these (e.g a glycoprotein, a lipoprotein, a peptide-nucleic acid etc).

[0053] In one particular embodiment, the CD96-inhibitory agent is an antibody or antibody fragment that binds CD96.

[0054] In a preferred form, the antibody or antibody fragment binds or interacts with an amino acid sequence of one or a plurality of external or extracellular immunoglobulin-like domains of CD96. For example, the antibody or antibody fragment may bind or interact with an amino acid sequence of: domain 1; domain 2; domain 3; domain 1 and domain 2; domain 1 and domain 3: domain 2 and domain 3; or domain 1, domain 2 and domain 3.

[0055] In one embodiment, the antibody binds or interacts with one or a plurality of external immunoglobulin-like domains of human CD96 isoform 2.

[0056] In one form the antibody binds CD96 and at least partly blocks or inhibits CD96 binding to CD155.

[0057] Antibodies may be polyclonal or monoclonal, native or recombinant. Antibody fragments include Fab and Fab'2 fragments, diabodies and single chain antibody fragments (e.g. scVs), although without limitation thereto. Antibodies and antibody fragments may be modified so as to be administrable to one species having being produced in, or originating from, another species without eliciting a deleterious immune response to the "foreign" antibody. In the context of humans, this is "humanization" of the antibody produced in, or originating from, another species. Such methods are well known in the art and generally involve recombinant "grafting" of non-human antibody complementarity determining regions (CDRs) onto a human antibody scaffold or backbone.

[0058] Suitably, the step of inhibiting or reducing CD96 activity in the mammal does not include killing CD96-expressing cells in the mammal. In this context, "killing" may refer to any antibody-mediated cytotoxic mechanism such as complement-mediated cytolysis and antibody-mediated cell-mediated cytotoxicity (ADCC), the latter typically mediated by natural killer (NK) cells, macrophages, neutrophils and eosinophils. In this regard, it may be advantageous to use antibody fragments lacking an Fc portion or having a mutated Fc portion.

[0059] The step of inhibiting or reducing CD96 activity in the mammal may be achieved or facilitated by administering a CD96-inhibitory agent to the mammal.

[0060] By "administering" is meant the introduction of the CD96-inhibitory agent into the mammal by a particular route. Suitably, a therapeutically effective amount of the CD96-inhibitory agent is administered to the mammal.

[0061] The term "therapeutically effective amount" describes a quantity of a specified agent sufficient to achieve a desired effect in a mammal being treated with that agent.

[0062] Generally, the method of the invention may be useful in reducing or relieving CD96-mediated immune inhibition, suppression or peripheral tolerance. Suitably, the method facilitates treatment or prevention of one or more diseases or conditions that are responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance.

[0063] As used herein, "treating" or "treat" or "treatment" refers to a therapeutic intervention that at least party eliminates or ameliorates one or more existing or previously identified symptoms of a disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance.

[0064] As used herein, "preventing" or "prevent" or "prevention" refers to a prophylactic treatment initiated prior to the onset of a symptom of a disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance, so as to at least partly or temporarily prevent the occurrence of the symptom.

[0065] Typically, the disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance is any disease or condition where enhanced or restored immune surveillance can benefit a subject suffering from the disease or condition. Such diseases and conditions may include those where persistence of the disease or condition can be controlled or suppressed by cell-mediated immunity. Non-limiting examples include cancers and viral infections. Particular viral infections contemplated by the invention include persistent viral infections such as caused by human immunodeficiency virus (HIV), Epstein Barr Virus (EBV), Herpes Simplex Virus (HSV inclusive of HSV1 and HSV2), Human Papillomavirus (HPV), Varicella zoster virus (VSV) and Cytomegalovirus (CMV), although without limitation thereto.

[0066] In a preferred embodiment, the method reduces or relieves immune inhibition in a mammal sufficient to treat or prevent cancer or cancer metastasis in the mammal. Suitably, the cancer may be any that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance. Cancers may be in the form of solid tumors, sarcomas, lymphomas, myelomas, carcinomas, melanomas, cytomas and meningiomas, although without limitation thereto. Non-limiting examples of cancers include cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, pituitary, parathyroid, prostate, salivary glands, skin, spleen, testis, thyroid, and uterus. Particular non-limiting examples of cancers include colon cancer, lung cancer and prostate cancer. In some embodiments, the cancer is a metastatic cancer, which is capable of migrating to another site, tissue or organ in the body and forming a tumor at that site, tissue or organ.

This may occur repeatedly over time. A particularly aggressive metastatic cancer contemplated by the invention is metastatic melanoma.

[0067] It will also be appreciated that the method of treatment or prevention of cancer may further include co-administration of one or more other therapeutic agents that facilitate cancer treatment or prevention. By way of example only, these include: chemotherapeutic agents such as paclitaxel, doxorubicin, methotrexate and cisplatin, although without limitation thereto; and/or biotherapeutic agents such as anti-PD-1 antibodies (e.g. Nivolumab) and anti-CTLA4 antibodies (e.g Ipilimumab), although without limitation thereto. Also contemplated are bi-specific antibodies that bind both CD96 and one or more other molecules including but not limited to PD-1 and CTLA4.

[0068] The one or more other agents that facilitate cancer treatment or prevention may be administered in combination with the CD96-inhibitory agent or be administered separately, as is well understood in the art.

[0069] In some embodiments, the CD96-inhibitory agent may be formulated alone or together with the one or more other agents is in the form of a pharmaceutical composition.

[0070] Suitable dosages of CD96-inhibitory agents, alone or together with other therapeutic agents, for mammalian administration, including human administration, may be readily determined by persons skilled in the art.

[0071] Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

[0072] Preferably, the pharmaceutically-acceptable carrier, diluent or excipient is suitable for administration to mammals, and more preferably, to humans.

[0073] By "pharmaceutically-acceptable carrier, diluent or excipient" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, diluents and excipients well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates, and pyrogen-free water.

[0074] A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. NJ USA, 1991).

[0075] Any safe route of administration may be employed for providing a subject with compositions comprising the CD96-inhibitory agent. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal, and the like may be employed.

[0076] A further aspect of the invention provides a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

[0077] The invention also provides a CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the aforementioned aspect.

[0078] The candidate molecule may be a protein (inclusive of peptides, antibodies and antibody fragments), a nucleic acid (inclusive of inhibitory RNA molecules such as ribozymes, RNAi, miRNA and siRNA, although without limitation thereto), a lipid, a carbohydrate, a small organic molecule or any combination of these (e.g a glycoprotein, a lipoprotein, a peptide-nucleic acid etc).

[0079] In some embodiments, the candidate modulator may be rationally designed or engineered de novo based on desired or predicted structural characteristics or features that indicate the candidate modulator could block or inhibit one or more biological activities of CD96, such as CD155 binding, intracellular signaling and/or IFN-γ production and/or secretion. In other embodiments, the candidate modulator may be identified by screening a library of molecules without initial selection based on desired or predicted structural characteristics or features that indicate the candidate modulator could block or inhibit one or more biological activities of CD96. Such libraries may comprise randomly generated or directed libraries of proteins, peptides, nucleic acids, recombinant antibodies or antibody fragments (e.g. phage display libraries), carbohydrates and/or lipids, libraries of naturally-occurring molecules and/or combinatorial libraries of synthetic organic molecules.

[0080] Non-limiting examples of techniques applicable to the design and/or screening of candidate modulators may employ X-ray crystallography, NMR spectroscopy, computer assisted screening of structural databases, computer-assisted modelling or biochemical or biophysical techniques which detect molecular binding interactions, as are well known in the art.

[0081] Biophysical and biochemical techniques which identify molecular interactions include competitive radioligand binding assays, co-immunoprecipitation, fluorescence-based assays including fluorescence resonance energy transfer (FRET) binding assays, electrophysiology, analytical ultracentrifugation, label transfer, chemical cross-linking, mass spectroscopy, microcalorimetry, surface plasmon resonance and optical biosensor-based methods, such as provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan et al., (John Wiley & Sons, 1997) Biochemical techniques such as two-hybrid and phage display screening methods are provided in Chapter 19 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan et al., (John Wiley & Sons, 1997).

[0082] Accordingly, an initial step of the method may include identifying a plurality of candidate molecules that are selected according to broad structural and/or functional attributes, such as an ability to bind CD96.

[0083] The method may include a further step that measures or detects a change in one or more biological activities of CD96 in response to the candidate molecule(s). These may include CD155 binding, the presence or absence of cell surface CD96, intracellular signaling, cytokine and/or chemokine production or secretion and/or protection from tumor challenge in an in vive model.

[0084] Inhibition of CD155 binding to CD96 by a candidate molecule may be determined by any of several techniques known in the art including competitive radioligand binding assays, surface plasmon resonance (e.g. BIACore™ analysis), co-immunoprecipitation and fluorescence-based

analysis of the ability of a candidate inhibitor to block CD155 binding to CD96 (such as by flow cytometry where CD155 is labeled with a fluorophore). Non-limiting examples of fluorophores include fluorescein isothiocyanate (FITC), allophycocyanin (APC), fluoroscein derivatives such as FAM and ROX, Texas Red, tetramethylrhodamine isothiocyanate (TRITL), R-Phycoerythrin (RPE), Alexa and Bodipy fluorophores, although without limitation thereto.

[0085] Alternatively, this fluorescence-based analysis could include FRET analysis (e.g. one protein coupled to a donor fluorophore, the other coupled to an acceptor fluorophore), although without limitation thereto.

[0086] In some embodiments, intracellular signaling may be measured directly at the level of CD96, such as by measuring recruitment of SH2 domain-containing PTPases by CD96 expressed by NK cells, or T cell subsets. A candidate molecule of the invention suitably prevents or reduces recruitment of SH2 domain-containing PTPases by CD96 in the presence of CD155. According to this embodiment, the candidate molecule may at least partly inhibit or prevent binding between CD96 and CD155, thereby at least partly inhibiting or preventing intracellular signaling by CD96, and/or at least partly inhibit or prevent intracellular signaling by CD96 despite CD155 binding.

[0087] In other embodiments, an effect of a candidate molecule on CD96 may be determined by measuring expression, production and/or secretion of one or more cytokines or chemokines by cells expressing CD96. Generally, changes in cytokine or chemokine expression production and/or secretion may be measured by RT-PCR of cytokine mRNA, measurement of cytokine or chemokine protein located intracellularly (e.g by immunocytochemistry using cytokine- or chemokine-specific antibodies) and/or measurement of secreted cytokines or chemokines such as by flow cytometric Cytokine Bead Array (such as commercially available from BD Biosciences), by ELISA using cytokine- or chemokine-specific antibodies and by bioassays that use cytokine- or chemokine-responsive cell lines to measure cytokines and/or chemokines secreted into cell supernatants. The cytokine may be any pro-inflammatory cytokine or chemokine inclusive of MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ, although without limitation thereto. Preferably, the cytokine is IFN-γ.

[0088] The effect of a candidate CD96 inhibitory agent may be upon CD96 expression, inclusive of cell surface expression and CD96 gene expression. It will be appreciated that in certain cells, such as NK cells although without limitation thereto, CD96 inhibitory agents may cause or facilitate a removal, loss and/or down-regulation of CD96 expression. In some embodiments, thus may include removal, loss and/or down-regulation of CD96 expression at the cell-surface. This may occur as a result of enhanced internalization or endocytosis of cell surface CD96 and/or by a down-regulation or suppression of CD96 gene expression. In particular embodiments, CD96 cell surface expression may be detected or measured by flow cytometry, immunoprecipitation, immunocytochemistry or immunohistochemistry, typically by way of an antibody or antibody fragment which binds CD96, as hereinbefore described. In particular embodiments, CD96 gene expression may be measured by nucleic acid sequence amplication (e.g. PCR inclusive of quantitative and semi-quantitaive PCR) or nucleic acid hybridization techniques such as Northern blotting.

[0089] Preferably, the CD96-inhibitory effect of a candidate molecule may be determined using an in vivo tumor challenge model. For example, a mouse model using CD96-expressing mice may be used to determine the ability of a candidate molecule to inhibit or prevent tumor formation and/or growth in response to an administered carcinogenic agent such as methycholanthrene (MCA). In another example, a mouse model using CD96-expressing mice may be used to determine the ability of a candidate molecule to inhibit or prevent tumor formation and/or growth in response to administration of tumor cells such as melanomas, colon adenocarcinomas, prostate carcinomas and mammary carcinomas, although without limitation thereto. Other mouse models may utilize mice that are predisposed to spontaneously forming tumors including but not limited to MMTV-polyoma, MT mammary cancer, DMBA/TPA induced skin cancer, p53 loss lymphoma/sarcoma and TRAMP Tg prostate cancer.

[0090] It will be understood that the method of this aspect may be performed iteratively, whereby multiple rounds of screening, design, and biological testing are performed. This may include where a candidate molecule is structurally modified before each round, thereby enabling "fine-tuning" of the candidate molecule.

[0091] It will also be appreciated that the method may be performed in a "high throughput", "automated" or "semi-automated" manner, particularly during early stages of candidate molecule identification and selection.

[0092] In a preferred embodiment, the candidate molecule is an antibody or antibody fragment. As hereinbefore described, antibodies may be polyclonal or monoclonal, native or recombinant. Antibody fragments include Fab and Fab'2 fragments, diabodies and single chain antibody fragments (e.g. scVs), although without limitation thereto. Well-known protocols applicable to antibody production, selection, purification and use may be found, for example, in Chapter 2 of Coligan et al., CURRENT PROTOCOLS IN IMMUNOLOGY (John Wiley & Sons NY, 1991-1994) and Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988, which are both herein incorporated by reference.

[0093] Polyclonal antibodies may be prepared for example by injecting CD96 or a fragment (e.g a peptide) thereof into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols that may be used are described for example in Coligan et al., CURRENT PROTOCOLS IN IMMUNOLOGY, supra, and in Harlow & Lane, 1988, supra.

[0094] Monoclonal antibodies may be produced using the standard method as for example, described in an article by Köhler & Milstein, 1975, Nature 256, 495, which is herein incorporated by reference, or by more recent modifications thereof as for example, described in Coligan et al., CURRENT PROTOCOLS IN IMMUNOLOGY, supra by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the isolated proteins, fragments, variants or derivatives of the invention. Suitably, the antibody or antibody fragment is suitable for administration to a human. In this context, as hereinbefore described the antibody or antibody fragment may be a "humanized" form of an antibody or antibody fragment produced in, or originating from, another species. Such methods are well known in the art and generally

involve recombinant "grafting" of non-human antibody complementarity determining regions (CDRs) onto a human antibody scaffold or backbone.

[0095] In a preferred embodiment, the antibody or antibody fragment does not kill CD96-expressing cells upon administration to a human. In this context, "killing" may refer to any antibody-mediated cytotoxic mechanism such as complement-mediated cytolysis and antibody-mediated cell-mediated cytotoxicity (ADCC), the latter typically mediated by natural killer (NK) cells, macrophages, neutrophils and eosinophils. In this regard, it may be advantageous to use antibody fragments lacking an Fc portion or having a human Fc portion (e.g a humanized antibody).

[0096] A CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the aforementioned aspect may be used according to the method of the first aspect (e.g as an anti-cancer agent and/or an anti-viral agent), preferably in the form of a pharmaceutical composition as hereinbefore described.

[0097] So that the invention may be readily understood and put into practical effect, reference is made to the following non-limiting examples.

EXAMPLES

Example 1

CD96 Binding to CD155 and the Effects of CD96 Inhibition and Knockout in Mouse Tumor Models

Materials and Methods

Mice

[0098] Wild Type C57BL/6 mice were purchased from the Walter and Eliza Hall Institute for Medical Research or ARC Animal Resource Centre. C57BL/6 $CD96^{-/-}$ mice were generated by Dr. Marco Colonna and Dr. Susan Gilfillan at the Washington University School of Medicine (St Louis, Mo., USA) as follows. A targeting construct designed to replace exons 1 and 2 of CD96, including the start site, with a MC1-neor gene flanked by loxP sites was electroporated into E14.1 (129P2/OlaHsd) embryonic stem cells (FIG. S1). Chimeras transmitting the targeted allele were obtained from two clones following injection into C57BL/6 blastocysts. Mice carrying the targeted allele were bred to C57BL6 mice expressing a Cre transgene under the CMV promoter to delete the MC1-neor gene (Schwenk et al., 1995). The CD96 deletion was backcrossed onto a C57BL/6 background, facilitated by a genome-wide screening of polymorphic microsatellite markers at 10-centiMorgan intervals at each generation. $CD96^{+/-}$ >99% C57BL/6 mice were intercrossed to generate the $CD96^{-/-}$ mice. DNAM-$1^{-/-}$ mice have already been described. DNAM-$1^{-/-}CD96^{-/-}$ were generated by intercrossing $CD96^{-/-}$ with DNAM-$1^{-/-}$ mice. Mice were bred and used between the ages of 6-14 weeks. All experiments were approved by animal ethics committee.

Cell Culture

[0099] B16F10, RM-1, 3LL, AT3, MC38 and YAC-1 cell lines were grown in complete RPMI Medium (Gibco, Invitrogen,) i.e supplemented with 10% FCS (Thermo Scientific), L-Glutamine (Gibco), Non-Essential Amino Acids, Sodium Pyruvate, HEPES (Gibco) and Penicillin/Streptomycin (Gibco), at 37° C. in 5% CO_2. For cytotoxicity assays and IL-12/IL-18 titration experiments, primary NK cells were harvested from the spleen, sorted using a mouse NK cell isolation kit (Miltenyi Biotec) and AutoMACS (Miltenyi Biotec), and subsequently cultured for 5 days in RPMI Medium supplemented with 10% FCS, L-Glutamine, Penicillin/Streptomycin, Non-Essential Amino Acids (Gibco), Sodium Pyruvate (Gibco), HEPES (Gibco), β-2-mercaptoethanol (Calbiochem), and 1000 IU/ml recombinant human IL-2 (Chiron Corporation). All cells were incubated at 37° C. in 5% CO_2.

In Vivo LPS Challenges

[0100] LPS (from *E. Coli* 0127:B8, Sigma) suspended in PBS was injected intraperitoneally into mice at the described doses. For survival curves, mice were checked hourly for symptoms of sepsis. Serum from these mice was taken at various time points by retro-orbital or cardiac bleeding for cytokine analysis. Spleens were also taken from mice at various time points to analyse receptor and ligand expression, and intracellular IFN-γ expression from NK cells.

In Vivo Tumor Challenges

[0101] Mouse B16F10 or B16-OVA melanomas, RM-1 prostate carcinoma, 3LL lung carcinoma, MC38-OVA^{dim} colon adenocarcinoma or AT3-OVA^{dim} mammary carcinoma, were injected into WT, DNAM-$1^{-/-}$, $CD96^{-/-}$, or DNAM-$1^{-/-}CD96^{-/-}$ mice subcutaneously or intravenously at the indicated doses and monitored for solid tumor growth or metastasis, respectively. Treatments were administered as indicated in the Figure legends. To monitor solid tumor growth, the area of the ensuing tumor was calculated by taking the length and width of palpable tumors by calipers and plotted against time. To monitor metastasis formation, 14 days after cells were injected, lungs were harvested, placed in Bouin's fixative, and metastases were counted using a dissection microscope.

MCA-Induced Fibrosarcoma

[0102] WT, DNAM-$1^{-/-}$, $CD96^{-/-}$ and DNAM-$1^{-/-}$ $CD96^{-/-}$ mice were injected subcutaneously on the right flank with various doses of MCA (5-400 μg, e.g. 100 g MCA) and were monitored over time for fibrosarcoma formation. In addition, some mice were treated with control antibody, depleted of NK cells by treatment with anti-asialoGM1 (Wako Chemicals; 100 μg injected i.p. at day −1, 0 and then weekly until week 8), neutralized for IFN-γ (H22, 250 μg injected i.p. at day −1, 0 and then weekly until week 8), for CD155, for DNAM-1 or CD96.

Dendritic Cells (BMDC): NK Cell Coculture Assays

[0103] BMDC were generated as described previously. Briefly, we harvested bone marrow cells from the femur and tibia of mice and cultured them in DMEM supplemented with 10% FCS, L-Glutamine, Penicillin/Streptomycin, Non-Essential Amino Acids, Sodium Pyruvate, β-2-mercaptoethanol and 250 ng/ml GM-CSF (eBioscience) for 6 days. WT or $CD96^{-/-}$ NK cells were harvested from the spleens and FACS sorted to purity by staining with NK1.1 (PK136) and TCRβ (H57-597) and CD3 (17A2) antibodies. NK cells were harvested on the day of the assay. For assay set up, 5×10^4 BMDM were plated in 96 well U bottom plates. NK cells were then added to the BMDM at varying titrations (2:1, 1:1, 0.5:1, and 0.25:1). BMDM only and NK only were always included in the assay as controls. Once all cells were plated, each well

was filled with the appropriate amount of media to yield equivalent volumes between wells. 100 ng/ml of LPS was then added to the wells for 2 h, followed by 5 mM purified ATP (Sigma) for 30 mins. This was performed at 37° C. in 5% CO_2. LPS only and ATP only controls were also included in the assay as controls. After 30 mins with ATP, supernatants were harvested and stored at −20° C. until analysed.

^{51}Cr Cytotoxicity Assays

[0104] Standard ^{51}Cr cytotoxicity assays were used to analyse the ability of WT and CD96$^{-/-}$ NK cells to kill targets. Briefly 20,000 targets labeled with 100 μCi of ^{51}Cr were added to V bottom plates and NK cells were then added to the targets at defined effector to target ratios. After 4 h at 37° C. in 5% CO_2, supernatants were harvested, and the level of ^{51}Cr was quantified by a gamma counter (Wallac Wizard). Percentage specific killing was determined using the formula (Sample Cr release-Spontaneous Cr release)/(Total Cr release-Spontaneous Cr release)×100.

Cytokine Detection

[0105] All cytokine detection in serum or supernatants except IL-18 was achieved by utilising Cytometric Bead Array (CBA) technology (BD Biosciences). Acquisition was completed using a Canto II or LSRII Flow Cytometric Analyser (BD Biosciences). Analysis was performed using the FCAP array software. IL-18 was detected by an ELISA according to manufacturer's instructions (MRL). For intracellular cytokine detection, isolated lymphocytes were obtained from the liver, stained for surface markers, fixed and permeabilised (BD Biosciences), and stained with an anti-IFN-γ antibody (XMG1.2).

Flow Cytometry Analysis and Sorting

[0106] Analysis of Immune Cell Homeostasis and CD96/CD155 expression: Various organs (lymph node, lung, spleen, bone marrow, and liver) were processed into single lymphocyte suspensions that included red blood cell lysis. Between 1×10^6 and 5×10^6 cells were initially subject to incubation with 2.4G2 to block non-specific Fc antibody binding before specific antibodies were utilised. To analyse NK cell homeostasis and IFN-γ production, the following antibodies were used: anti mouse-NK1.1, -TCRβ, -CD27 (LG.7F9), -CD11b (M1/70), and -IFN-γ. For T cells: anti mouse-TCRβ, -CD8 (53-6.7), and -CD4 (RM4-5). For B cells: anti mouse-B220 (RA3-6B2), -CD19 (1D3). For NKT cells: mouse CD1d tetramer loaded with α-galactosylceramide (kindly provided by Professor Dale Godfrey, University of Melbourne), anti mouse-TCRβ or -CD3, -CD4, and -NK1.1. For macrophages: anti mouse-F4/80 (BM8) and -CD11b. For neutrophils: anti mouse-Ly6G (1A8) and -CD11b. For conventional DC: anti mouse-MHC II (M5/114.15.2) and -CD11c (N418). For 76 T cells: anti mouse-γδ TCR (GL3) and -CD3. To analyse CD96 and CD155 expression, the specific cell type of interest was gated upon using the above antibody cocktails along with anti mouse-CD96 (3.3.3) or anti mouse-CD155 (4.24.3). Acquisition was performed using an LSR II, or Canto II flow cytometric analyser (BD Biosciences). Analysis was achieved using Flowjo (Treestar).

Cell Sorting

[0107] Naïve NK cells and macrophages from the spleen were prepared and stained for as described above. These cells were then sorted to purity using an Aria II FACS sorter (BD Biosciences).

Statistical Analysis

[0108] Statistical analysis was achieved using Graphpad Prism Software. Data was considered to be statistically significant where the p value was equal to or less than 0.05. Statistical tests used were the unpaired Student's t test, Mann Whitney t test, and the Mantel-Cox test for survival. The appropriate test used is defined in the Figure legends.

Results

[0109] CD96 competes with DNAM-1 for CD155 binding (FIG. **1**) and CD96 engagement by CD155 down-regulates NK cell production of IFNγ (FIG. **2**). CD96 limits NK cell-dependent tumor immunosurveillance in MCA-treated mice and promotes experimental B16F10 lung metastasis (FIG. **3**).

[0110] The data in FIG. **4** show that anti-CD96 mAb has single agent activity (i.e without anti-PD1 treatment) while also enhancing the anti-tumor responses of anti-PD1. Anti-CD96 mAb treatment also enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy (FIGS. **5** & **7**) which is consistent with FIG. **6** where enhanced anti-tumor responses to Doxorubicin (DOX) chemotherapy were observed in host with CD96 deficiency. Referring to FIGS. **8** & **9**, given early or late, anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs and shows a particularly strong synergy with anti-PD-1.

[0111] The effect of CD96 in promotion of tumour metastasis was also investigated. In FIG. **10**, regulation of B16F10 lung metastasis was investigated in C57BL/6 wild type (WT), DNAM-1$^{-/-}$, CD96$^{-/-}$, and DNAM-1$^{-/-}$CD96$^{-/-}$ mice In FIG. **11**, host CD96 promoted RM-1 lung metastasis and in FIG. **12**, host CD96 promoted 3LL lung metastasis FIG. **13** shows that anti-CD96 mAb suppresses B16F10 lung metastasis, alone and in combination with a T cell checkpoint blockade. In FIG. **14**, anti-CD96 mAb suppresses RM-1 lung metastasis, alone and in combination with T cell checkpoint blockade. In FIG. **15**, anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs against MC38 colon tumors and shows a particularly strong synergy with anti-PD-1.

Example 2

Screening Assays for Identifying Anti-CD96 Antibodies

Introduction

[0112] The following assays may be used to identify antibodies useful in the invention. The first assay would be used to identify human antibodies capable of blocking or inhibiting binding between human CD96 and human CD155. The second assay may be used to test whether or not the identified antibodies cause antibody-dependent cell-mediated cytotoxicity (ADCC). The third assay can then be applied to lead candidates and involves determining whether or not a human CD96 antibody can modulate human lymphocyte effector function.

Materials and Methods

Assay 1: CD96 Binding to CD155

[0113] The ability of candidate anti-CD96 antibodies to prevent the binding of CD155 to the cell surface of CD96 expressing cells (such as NK cells) will be tested as follows.

Recombinant Human CD155 fused to the C terminal Fc region of human IgG1 (such as CD155-Fc available from Sino Biological) will be labeled with a fluorophore such as Alexa Fluor 647 (AF647) using Zenon Human IgG Labeling kit (Molecular Probe) accordingly to the manufacturer's instructions. NK cells or other CD96-expressing cells freshly isolated from the peripheral blood of healthy donors will be incubated with AF647 labeled CD155-Fc in the presence of anti-CD96 or control Ig at different concentrations (The cells will be harvested and the cell surface binding of AF647-CD155-Fc will be tested by flow cytometry). Antibodies that prevent binding of CD155 cells to CD96-expressing cells will be identified by their ability to block binding of CD155-Fc to CD96-expressing cells.

Assay 2: ADCC Assay

[0114] The survival of immune cells (such as NK cells and/or T cells) in the presence of anti-CD96 antibodies will be analyzed as follows. The peripheral blood immune cells from healthy donors will be isolated by Ficoll gradient separation. Immune cells will be plated in 96 well plates in the presence of human IL-2 at an appropriate dosage and increasing concentrations of anti-CD96 mAbs. The survival as well as the percentages of CD96 expressing cells (such as NK cells and/or T cells) will be analyzed over time by flow cytometry. A non-limiting example of a suitable commercially available kit for this assay is the Annexin V Apoptosis Detection Kit.

Assay 3: Assay for Modulation of Human Leukocyte Effector Function by Human CD96 Antibodies

[0115] Fresh blood samples will be collected from healthy donors. Peripheral blood mononuclear cells (PBMC) will be prepared on a Ficoll-Paque density gradient by centrifugation. Highly pure CD3-CD56+NK cells will be obtained from PBMC by magnetically activated cell sorting. To analyze the ability of CD96 to impact human NK cell production of IFN-γ, 96 well U bottom plates will be coated overnight at 4° C. with recombinant Human CD155-Fc chimera (Sino Biological Inc.; 0.25 μg/well) or with non-relevant human IgG1 antibodies. Freshly purified human NK cells will then be plated in complete RMP1 media supplemented with Human IL-12, IL-18 and optionally IL-15 for 24 h and the intracellular content and the level of IFN-γ in the supernatant will be analysed in the different cultures. Alternatively, human NK cells will be stimulated for 24 h in wells coated with anti-NKG2D, anti-NKp46, anti-NKp30 or anti-CD16 antibodies to analyze the ability of CD96 signalling to interact with other NK cells receptors. The anti-human CD96 antibodies to be tested or control antibodies will be added to the cultures prior to the cytokines or antibodies above to confirm the ability of these test anti-human CD96 antibodies to enhance the IFNγ production of the human NK cells. Statistical increases in IFNγ production above the control would be considered significant.

Example 3

Assay for Modulation of Mouse NK Cell Function by Anti-CD96 Antibodies

[0116] Additional anti-mouse CD96 antibodies would also be screened for the ability to modulate CD96 signalling activity. To analyse mouse NK cell production of IFN-γ, 96 well U bottom plates would be coated overnight at 4° C. with recombinant mouse CD155-Fc chimera (Sino Biological Inc.; 0.25 μg/well) or with non-relevant human IgG1 antibodies. After three washes with PBS, freshly purified NK cells from the indicated mouse strains would be plated in complete RMP1 media supplemented with mouse IL-12 (ebiosciences; 25-100 μg/ml) and mouse IL-18 (R&D; 50 ng/ml) for 24 b. Alternatively, IL-2-activated NK cells would be stimulated for 6 h in wells coated with anti-NK1.1 (PK136; 0.125 μg/well). At different time points, anti-mouse CD96 antibodies (50-200 μg/ml) or control antibodies will be added to determine whether these enhance NK cell IFN-g production as measured by CBA analysis.

Example 4

Production of Mouse and Human Anti-CD96 Antibodies

[0117] Human CD96 is a transmembrane protein that exists in two isoforms. Isoform 1 has been detected in acute myeloid leukaemia and includes additional amino acids compared with isoform 2. Isoform 2 is the common form in humans and the predicted domain architecture of isoform 2 has three (3) external immunoglobulin-like domains (domains 1, 2 and 3), as listed in Table 1. Antibodies against isoform 2 are preferred for use in the present invention. The nucleic and amino acid sequences of isoform 2 are given in the NCBI consensus sequence number CCDS2958.1 (SEQ ID NOS: 1 and 2 respectively).

Human CD96 CDNA isoform 2.

SEQ ID NO: 1

ATGGAGAAAAAATGGAAATACTGTGCTGTCTATTACATCATCCAGATACA

TTTTGTCAAGGGAGTTTGGGAAAAAACAGTCAACACAGAAGAAAATGTTT

ATGCTACACTTGGCTCTGATGTCAACCTGACCTGCCAAACACAGACAGTA

GGCTTCTTCGTGCAGATGCAATGGTCCAAGGTCACCAATAAGATAGACCT

GATTGCTGTCTATCATCCCCAATACGGCTTCTACTGTGCCTATGGGAGAC

CCTGTGAGTCACTTGTGACTTTCACAGAAACTCCTGAGAATGGGTCAAAA

TGGACTCTGCACTTAAGGAATATGTCTTGTTCAGTCAGTGGAAGGTACGA

GTGTATGCTTGTTCTGTATCCAGAGGGCATTCAGACTAAAATCTACAACC

TTCTCATTCAGACACACGTTACAGCAGATGAATGGAACAGCAACCATACG

ATAGAAATAGAGATAAATCAGACTCTGGAAATACCATGCTTTCAAAATAG

CTCCTCAAAAATTTCATCTGAGTTCACCTATGCATGGTCGGTGGAGGATA

ATGGAACTCAGGAAACACTTATCTCCCAAAATCACCTCATCAGCAATTCC

ACATTACTTAAAGATAGAGTCAAGCTTGGTACAGACTACAGACTCCACCT

CTCTCCAGTCCAAATCTTCGATGATGGGCGGAAGTTCTCTTGCCACATTA

GAGTCGGTCCTAACAAAATCTTGAGGAGCTCCACCACAGTCAAGGTTTTT

GCTAAACCAGAAATCCCTGTGATTGTGGAAAATAACTCCACGGATGTCTT

GGTAGAGAGAAGATTTACCTGCTTACTAAAGAATGTATTTCCCAAAGCAA

ATATCACATGGTTTATAGATGGAAGTTTTCTTCATGATGAAAAAGAAGGA

ATATATATTACTAATGAAGAGAGAAAAGGCAAAGATGGATTTTTGGAACT

GAAGTCTGTTTTAACAAGGGTACATAGTAATAAACCAGCCCAATCAGACA

-continued

ACTTGACCATTTGGTGTATGGCTCTGTCTCCAGTCCCAGGAAATAAAGTG

TGGAACATCTCATCAGAAAAGATCACTTTTCTCTTAGGTTCTGAAATTTC

CTCAACAGACCCTCCACTGAGTGTTACAGAATCTACCCTTGACACCCAAC

CTTCTCCAGCCAGCAGTGTATCTCCTGCAAGATATCCAGCTACATCTTCA

GTGACCCTTGTAGATGTGAGTGCCTTGAGGCCAAACACCACTCCTCAACC

CAGCAATTCCAGTATGACTACCCGAGGCTTCAACTATCCCTGGACCTCCA

GTGGGACAGATACCAAAAAATCAGTTTCACGGATACCTAGTGAAACATAC

AGTTCATCCCCGTCAGGTGCAGGCTCAACACTTCATGACAATGTCTTTAC

CAGCACAGCCAGAGCATTTTCAGAAGTCCCCACAACTGCCAATGGATCTA

CGAAAACTAATCACGTCCATATCACTGGTATTGTGGTCAATAAGCCCAAA

GATGGAATGTCCTGGCCAGTGATTGTAGCAGCTTTACTCTTTTGCTGCAT

GATATTGTTTGGTCTTGGAGTGAGAAAATGGTGTCAGTACCAAAAAGAAA

TAATGGAAAGACCTCCACCTTTCAAGCCACCACCACCTCCCATCAAGTAC

ACTTGCATTCAAGAGCCCAACGAAAGTGATCTGCCTTATCATGAGATGGA

GACCCTCTAG

Human CD96 protein sequence isoform 2

SEQ ID NO: 2

MEKKWKYCAVYYIIQIHFVKGVWEKTVNTEENVYATLGSDVNLTCQTQTV

GFFVQMQWSKVTNKIDLIAVYHPQYGFYCAYGRPCESLVTFTETPENGSK

WTLHLRNMSCSVSGRYECMLVLYPEGIQTKIYNLLIQTHVTADEWNSNHT

IEIEINQTLEIPCFQNSSSKISSEFTYAWSVEDNGTQETLISQNHLISNS

TLLKDRVKLGTDYRLHLSPVQIFDDGRKFSCHIRVGPNKILRSSTTVKVF

AKPEIPVIVENNSTDVLVERRFTCLLKNVFPKANITWFIDGSFLHDEKEG

IYITNEERKGKDGFLELKSVLTRVHSNKPAQSDNLTIWCMALSPVPGNKV

WNISSEKITFLLGSEISSTDPPLSVTESTLDTQPSPASSVSPARYPATSS

VTLVDVSALRPNTTPQPSNSSMTTRGFNYPWTSSGTDTKKSVSRIPSETY

SSSPSGAGSTLHDNVFTSTARAFSEVPTTANGSTKTNHVHITGIVVNKPK

DGMSWPVIVAALLFCCMILFGLGVRKWCQYQKEIMERPPPFKPPPPPIKY

TCIQEPNESDLPYHEMETL

[0118] The Mouse CD96 protein is also a transmembrane protein but only a single transcript/isoform is known in the mouse as shown in SEQ ID NOs. 3 and 4.

Mouse cDNA

SEQ ID NO: 3

ATGGGGAGAAAATGGACATATTGTGTTGTTTACACCATCATCCAGATACA

GTTTTTCAGGGGAGTGTGGGAAGAGCTATTCAATGTTGGAGATGACGTGT

ATGCTCTACCTGGTTCTGACATCAACTTGACCTGCCAAACAAAGGAGAAA

AACTTCTTGGTACAGATGCAGTGGTCCAAGGTCACAGATAAGAATGACAT

GATTGCTCTTTATCATCCCCAATATGGCCTCTACTGTGGGCAGGAGCATG

CCTGTGAGTCACAAGTGGCTGCCACAGAAACTGAGAAGGGTGTAACAAAT

TGGACTCTGTACTTAAGGAATATCTCTTCTGCCCTGGGTGGAAAGTATGA

-continued

GTGCATCTTTACTCTGTATCCAGAAGGCATCAAGACTACAGTCTACAACC

TCATTGTGGAACCCTATACACAAGATGAACACAACTATACAATAGAAATA

GAGACAAATCGGACTCTGGAAATACCATGCTTTCAAAATACCTCCTCAGA

AATTCCACCTAGGTTCACCTTTTCATGGTTGGTGGAGAAAGATGGAGTGG

AAGAAGTTCTCTTCACCCACCATCACCACGTCAACAATTCCACATCATTT

AAAGGCAGAATCAGGCTGGGTGGAGACTATAGACTCCACCTCTCCCCAGT

CCAAATCCAAGACGATGGCAGGACATTCTCTTGCCATCTGACTGTCAATC

CTCTCAAAGCCTGGAAGATGTCCACCACAGTCAAGGTTTTTGCTAAACCA

GAAATCCTCATGACTGTGGAAAACAGCACCATGGATGTCTTAGGAGAGAG

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GGTTTATAGACGGAAGATTTCTTCAAGGCAACGAAGAAGGAATATACATT

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Mouse CD96 amino acid sequence

SEQ ID NO: 4

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VL

TABLE 1

External immunoglobulin like domains of CD96 from Interpro predictions

	Human CD96 isoform 2 residue numbers	Mouse CD96 residue numbers
Domain 1	30-137	30-137
Domain 2	148-250	145-247
Domain 3	253-359	250-355

[0119] The external domains of the mouse and human CD96 proteins will be cloned into appropriate expression constructs for expression in mammalian cells such as human embryonic kidney cells. Suitable expression constructs typically include a CMV promoter to drive expression of the CD96 gene fragment. Following transfection into mammalian cells the protein will be expressed under suitable culture conditions before being purified prior to antibody production.

[0120] Four CD96 knockout mice will be immunized with the mouse CD96 external domain protein and likewise four CD96 knockout mice will be immunized with the purified human CD96 external domain protein. Immunisations will be made at approximately three times at four week intervals. The mice will be bled 10-12 days following the third immunization and the sera titrated on the screening antigens by ELISA. The mice with the highest antibody titres would be used for fusion, otherwise if mice have not responded adequately further immunisations would be undertaken. Selected hybridomas will be cloned and mAbs purified from each clone before the individual human or mouse mAbs are screened using the screening assays of Examples 2 or 3 respectively. Isotyping of clones would optionally be undertaken in order to identify antibodies that are less likely or unable to induce ADCC, such as IgG2 and IgG4 antibodies. Approximately 20 antibodies against human CD96 and 20 antibodies against mouse CD96 would be obtained as listed in Table 1 of Example 4.

Example 5

Screening of Mouse and Human Anti-CD96 Antibodies

[0121] Approximately 20 each of anti-mouse CD96 and anti-human CD96 monoclonal antibodies would be obtained as described in Example 4. The anti-human CD96 antibodies would be screened for the ability to modulate CD96 signalling activity using the Human NK cell assays described in Example 2. An additional four commercially available anti-human CD96 antibodies (1C8, NK92.39, 3H8, MAA6359) will also be screened for the ability to modulate CD96 signalling. Anti-mouse CD96 mAbs would be also screened for their ability to modulate NK cell function as described in Example 3.

[0122] As can occur with antibodies, not all antibodies are expected to have a useful effect on a given target. Accordingly, CD96 signalling for each antibody will be assessed using the human or mouse NK cell assays to determine which antibodies have an effect upon CD96 signalling.

Preliminary Results Using the Human NK Cell Assay

[0123] Using the human NK cell assay described in Example 2, the NK92.39 human CD96 mAb was found to increase the levels of IFN-γ in human NK cells as shown in FIGS. **17**A and **17**B. This result indicates that antibodies against the human CD96 receptor can be effective in increasing IFN-γ production in human NK cells.

Example 6

Anti-Mouse CD96 Antibody Testing in Cancer Models

[0124] Anti-mouse CD96 antibodies that are found to be active in modulating CD96 signalling will be tested for their ability to relieve immune inhibition, reduce CD96 activity and/or enhance or restore immune surveillance in mouse cancer models.

[0125] Approximately 10 active anti-mouse CD96 antibodies will be individually tested in approximately 5-7 cancer models using In viva tumor challenges, the same or similar to those used in Example 1.

[0126] The efficacy of each CD96 antibody against a respective tumour and/or in a tumour model may vary and some may be more responsive to treatment than others. Non-limiting examples of tumours and tumour models to be tested may include breast, prostate, lung, melanoma, colorectal, pancreatic, endometrial, kidney, bowel, gastric, oesophageal, leukaemia, lymphoma, ovarian, bladder and brain cancers including primary tumours and/or metastases of the aforementioned cancers.

Example 7

Human CD96-Antibody Binding Studies

[0127] A subset of anti-human CD96 antibodies will be examined further to determine which domains of CD96 are bound by effective CD96 antibodies. As discussed in Example 3 above, isoform 2 of CD96 includes three external domains (Domains 1, 2 and 3).

[0128] Antibodies will be bound to the CD96 protein and the specific CD96 protein residues bound will be determined using hydrogen/deuterium exchange and mass spectrometry.[23,24] Alternatively, other methods may be employed to probe antibody-CD96 binding sites such as X-ray crystallography, site directed mutagenesis or other methods known in the art.

[0129] It is anticipated that effective antibodies may bind one or multiple external domains of the CD96 protein. For example anti-CD96 antibodies that modulate human NK cell function may bind any of the following combinations of external CD96 domains, with each possible single or combination binding domain shown in brackets: (1), (2), (3), (1,2), (1,3), (2,3), (1,2,3).

Example 8

CD96 Roles in Both NK and T Cell Function

[0130] Natural killer (NK) cells are innate lymphocytes which may be critical to limit early tumour growth and metastasis while T cells may be more important in the control of established and primary tumours. CD96 is a checkpoint immunomodulator that can affect both NK and T cell function.

[0131] Primary tumors were used to examine the role of CD96 in T cells. The AT3-OVA^{dim} model was used where CD8$^+$T effector cells are known to naturally control tumor growth. AT3-OVA^{dim} mammary carcinoma (1×10^6 cells) were injected subcutaneously. Mice were then monitored for tumor growth and measurements made with a caliper square as the product of two perpendicular diameters (mm^2).

[0132] Anti-CD96 treatment greatly reduced the rate of tumour growth and this beneficial effect could be removed by depletion of CD4 and CD8 T cells using anti CD4/CD8 antibody or by treatment with anti-IFN-γ (FIGS. **16**A and **16**B). This demonstrates that anti-CD96 mAb critically required CD8$^+$ T cells and IFN-γ for full anti-tumour activity in this particular tumour model.

Example 9

Loss of CD96 from the NK Cell Surface after aCD96 mAb Binding

[0133] The mechanistic/signaling effects that may occur upon antibody binding to CD96 may also reveal functional information about the effects of CD96-CD155 binding. To further investigate this, total NK cells were purified from human peripheral blood mononuclear cells (PBMCs) by negative selection using human NK cell isolation kit (Miltenyi Biotec.). Isolated NK cells were then labeled with carboxyfluorescein diacetate succinmidyl ester (CFSE; Biolegend) to measurecellular proliferation. CFSE-labeled NK cells were plated in 96 well U-bottom plate at 5×10^4 cells/well and stimulated with recombinant IL-2 at indicated concentrations (10 units/ml and 25 units/ml), in the presence of control IgG or anti-human CD96 mAb (clone NK92-39) at 30 μg/ml. NK cells were assessed for changes in proliferation or the presence/absence of surface CD96 at day 3 and 6 using BD FACS Canto II (BD Biosciences) and analysis was carried out using FlowJo (Tree Star) (FIGS. **18** (A) and (B). Anti-CD96 binding to CD96 had no effect on NK cell proliferation but appeared to greatly reduce the level of CD96 on the cell surface by day 6, either by internalization of CD96 following mAb binding or possibly via a reduction in CD96 expression.

[0134] Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

[0135] All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

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        355                 360                 365

Pro Thr Val Thr Gly Ser Thr Leu Gly Thr Gln Pro Phe Ser Asp Ala
    370                 375                 380

Gly Val Ser Pro Thr Gly Tyr Leu Ala Thr Pro Ser Val Thr Ile Val
385                 390                 395                 400

Asp Glu Asn Gly Leu Thr Pro Asp Ala Thr Pro Gln Thr Ser Asn Ser
                405                 410                 415

Ser Met Thr Thr Lys Asp Gly Asn Tyr Leu Glu Ala Ser Ser Gly Thr
            420                 425                 430

Asp Ala Lys Asn Ser Ser Arg Ala Ala Ala Ser Ser Lys Ser Gly Ser
        435                 440                 445

Trp Pro Phe Pro Phe Thr Ser Pro Pro Glu Trp His Ser Leu Pro Gly
    450                 455                 460

Thr Ser Thr Gly Pro Gln Glu Pro Asp Ser Pro Val Ser Trp Ile Pro
465                 470                 475                 480

Ser Glu Val His Thr Ser Ala Pro Leu Asp Ala Ser Leu Ala Pro His
                485                 490                 495

Asp Thr Ile Ile Ser Thr Thr Thr Glu Phe Pro Asn Val Leu Thr Thr
            500                 505                 510

Ala Asn Gly Thr Thr Lys Ile Asp His Gly Pro Ile Thr Ser Ile Ile
        515                 520                 525
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Val Asn Gln Pro Ser Asp Gly Met Ser Trp Pro Val Leu Val Ala Ala
    530                 535                 540

Leu Leu Phe Phe Cys Thr Leu Leu Phe Gly Leu Gly Val Arg Lys Trp
545                 550                 555                 560

Tyr Arg Tyr Gln Asn Glu Ile Met Glu Arg Pro Pro Pro Phe Lys Pro
                565                 570                 575

Pro Pro Pro Pro Ile Lys Tyr Thr Tyr Ile Gln Glu Pro Ile Gly Cys
            580                 585                 590

Asp Leu Cys Cys His Glu Met Glu Val Leu
        595                 600
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1. A method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

2. The method of claim **1**, wherein the step of at least partly inhibiting or reducing CD96 activity in the mammal does not include, or at least depend upon, killing of CD96-expressing cells in the mammal.

3. The method of claim **1**, wherein the step of at least partly inhibiting or reducing CD96 activity in the mammal includes administering a CD96-inhibitory agent to the mammal.

4. The method of claim **3**, wherein the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of CD96.

5. The method of claim **3**, wherein the CD96-inhibitory agent binds or interacts with: one or a plurality of external immunoglobulin-like domains of CD96 selected from the group consisting of: domain 1; domain 2; domain 3; domain 1 and domain 2; domain 1 and domain 3: domain 2 and domain 3; and domain 1, domain 2 and domain 3.

6. The method of claim **4**, wherein the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of human CD96 isoform 2 (SEQ ID NO:2).

7. The method of claim **3**, wherein the CD96-inhibitory agent at least partly blocks or inhibits CD96 binding to CD155 and/or intracellular signaling by CD96.

8. The method of claim **7**, wherein the CD96-inhibitory agent is an anti-CD96 antibody or antibody fragment.

9. The method of claim **1** which includes administering one or more other therapeutic agents.

10. The method of claim **9**, wherein the one or more other therapeutic agents include a chemotherapeutic agent and one or more antibodies or antibody fragments that bind PD1 and/or CTLA4.

11. The method of claim **1**, which increases or enhances cytokine and/or chemokine expression and/or secretion by one or more cells in the mammal.

12. The method of claim **11**, wherein the cytokine and/or chemokines include MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ,

13. The method of claim **12**, wherein the cytokine is interferon γ (IFN-γ).

14. The method of claim **11**, wherein the one or more cells are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδ T cells and NK T cells and natural killer (NK) cells.

15. The method of claim **1**, which treats or prevents cancer or cancer metastasis in the mammal.

16. The method of claim **1**, which treats or prevents a viral infection in the mammal.

17. The method of claim **1**, wherein the mammal is a human.

18. A method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

19. The method of claim **18** wherein the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of CD96.

20. The method of claim **19**, wherein the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of CD96 selected from the group consisting of: domain 1; domain 2; domain 3; domain 1 and domain 2; domain 1 and domain 3: domain 2 and domain 3; and domain 1, domain 2 and domain 3.

21. The method of claim **19** wherein the CD96-inhibitory agent binds or interacts with one or a plurality of external immunoglobulin-like domains of human CD96 isoform 2 (SEQ ID NO:2).

22. The method of claim **18**, wherein the CD96-inhibitory agent is an antibody or antibody fragment.

23. The method of claim **18**, wherein the CD96-inhibitory agent is an anti-cancer agent.

24. The method of claim **18**, wherein the CD96-inhibitory agent is an anti-viral agent.

25. The method of claim **18**, wherein the mammal is a human.

26. A CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the method of claim **18**.

27. (canceled)

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